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Studies on influenza virus replicon particle vaccines in pigs

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ABSTRACT

An influenza A virus (IAV) vaccine that provides better cross protection to pigs against heterologous strain infection and overrides maternal antibodies interference is desirable. A replicon particle (RP) platform has been developed for influenza vaccine for swine use by expressing influenza proteins.

Chapter 2 presented a review of influenza virus infection in swine, including a discussion of current vaccine approaches and techniques used for novel vaccine development.

The first animal study (Chapter 3), composing two experiments, demonstrated the efficacy of intranasal (IN) administration of RP expressing hemagglutin (HA) protein (HA RP) of Pandemic H1N1 (A/CA/04/2009) strain. Prime/boost HA RP vaccination was administered IN/IN to pigs, with IM/IM administration as positive control and sham vaccination as negative control (five pigs each group). All pigs were challenged by homologous challenge following two doses vaccination. Pigs were not protected by IN/IN administration of RP against IAV challenge. Virus titers and pneumonia were not reduced significantly in RP IN/IN administered group, compared with control group. In the second experiment, pigs were administered with one dose HA RP vaccine IM or IN, or with a combination of IN/IM routes with an interval of three weeks. At most, two pigs at 2 DPC and 3 DPC, and no pigs at 3 DPC to 5DPC in IN/IM or one dose IM administered pigs were isolated with live virus, while all five pigs were detected with live virus from one dose RP IN administered pigs and sham vaccinated pigs. We also found that one dose IM and

combination of IN/IM vaccination with HA RP reduced pneumonia significantly compared with sham vaccinated group, contrary to one dose IN vaccination.

In the second animal study (Chapter 4), the immunogenicity of HA RPs of a H3N2 strain and a delta1 strain at varying doses [1×10^7 (infectious unit) IU to 5×10^5 IU], was evaluated. Two H3N2 RP vaccinated groups with 10^6 IU or 5×10^5 IU RP as negative control, and two delta1 RP vaccinated groups with 10^6 IU or 5×10^5 IU RP were all challenged with delta1 IAV virus homologous to delta1 RP. RPs at 5×10^5 IU or higher doses tested in this study were sufficient to induce hemagglutinnin inhibition (HI) detectable antibodies. HI titers were greater at increasing vaccine doses. Delta1 RP 10^6 IU or 5×10^5 IU doses, which induced HI antibodies with mean titers equal to or lower than 20, partially prevent homologous infection by eliminating virus replication in lung at day 5 post challenge. However, compared with H3N2 RP vaccinated pigs, decreased lung lesions observed in delta1 RP vaccinated pigs were not statistically significant.

CHAPTER 1. INTRODUCTION: THESIS ORGANIZATION

This thesis consists five chapters. Chapter 1 is an introduction of the thesis organization. Chapter 2 is a review of literature, “Vaccine development for protecting swine against influenza virus”. The manuscript will be submitted to Animal Health Research Reviews. Chapter 3 are composed of two animal experiments, evaluating the efficacy of replicon particles (RP) by immunizing pigs intranasally. Chapter 4 determined RP vaccine of delta1 subtype dose, hemagglutinin inhibition and challenge responses. The last chapter is a general conclusion of the entire thesis research.

CHAPTER 2. VACCINE DEVELOPMENT FOR PROTECTING SWINE AGAINST INFLUENZA VIRUS

A manuscript to be submitted to *Animal Health Research Reviews*

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Abstract

Influenza virus infects a wide variety of species including humans, pigs, horses, sea mammals and birds. Weight loss caused by influenza infection and/or co-infection with other infectious agents results in significant financial loss in swine herds. The emergence of Pandemic H1N1 (A/CA/04/2009/H1N1), which infects both humans and livestock caused pandemic disease and brought about a concerning public health threat. Influenza virus contains eight single stranded, negative-sense RNA genome segments. This genetic structure allows the virus to evolve rapidly by antigenic drift and shift. Antigen specific antibodies induced by current vaccines provide limited cross protection to heterologous challenge. In pigs, this presents a major obstacle for vaccine development. Different strategies are under development to produce vaccines that provide better cross-protection for swine. Moreover, overriding interfering maternal antibodies is another goal for influenza vaccines in order to immunize piglets at an early age. Herein, we present a review of influenza virus infection in swine, including a discussion of current vaccine approaches and techniques used for novel vaccine development.

Influenza in swine

Influenza is a zoonotic disease caused by influenza virus which infects a wide variety of species including humans, pigs, horses, sea mammals and birds. Influenza virus was first isolated in the United States in 1930 (Shope 1931) and transmission between species happens occasionally (Vincent et al. 2008a). Sero-archeological studies of human samples from 1918 to 1920 showed the original causative virus of the 1918 pandemic flu was closely related to influenza virus A/Swine/Iowa/30 (H1N1) strain which is now referred to as classic H1N1 (cH1N1) (Webster 1999).

Viral characteristics

Influenza virus belongs to the family *Orthomyxoviridae*. Three types of influenza virus, type A, B and C, are grouped based on antigenic characteristics of the nucleoprotein (NP) (Mahy, 1997; Alexander and Brown, 2000a). Pathogenic influenza viruses in domestic animals are type A viruses (Maclachlan and Dubovi, 2011). Influenza A viruses (IAV) are enveloped with a size of around 80-120 nm and possess eight single stranded, negative-sense RNA genome segments packaged within virions (Maclachlan and Dubovi, 2011). Eleven proteins are encoded by these eight segments, including hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1) and 2 (M2), polymerase basic 1(PB1) and 2 (PB2), NP, polymerase acidic (PA), PB1-F2 and nonstructural protein 1 (NS1) and 2 (NS2) (Maclachlan and Dubovi, 2011). Structural proteins HA, NA, M1 and M2 form the envelope of IAV with the cellular lipid bilayer. HA protein plays a critical role during IAV cell entry. HA attachment to permissive cell sialic acid receptor on the plasma membrane initiates virus

entry cells via receptor-mediated endocytosis (Murphy et al., 1999). NA also binds to cell receptor sialic acid. During mature progeny virus detaching from host cells, the binding of NA and sialic acid on the same host cell prevents progeny virus self aggregation back to the same host cell mediated by HA (Grienke et al., 2012). In addition, the surface glycoproteins HA and NA induce protective specific immune responses in the host, but are not highly conserved (Alexander and Brown, 2000). At present, 16 distinct HA antigenic subtypes and 9 NA subtypes are identified (Bouvier and Palese, 2008) allowing further sub-typing according to the combination of HA and NA proteins present on the virion surface.

PB1, PB2 and PA form a trimeric RNA polymerase complex that binds one end of RNA segments and forms ribonucleoprotein (RNP) complexes with NP (Klumpp et al., 1997). RNP is required to transcribe positive strand mRNA and complementary cRNA, because negative strand RNAs cannot serve as translation templates directly (Baltimore et al., 1970; Conzelmann, 1998). Segments 7 and 8 encode two proteins respectively (M1and M2, NS1and NS2) by differential splicing of mRNAs (Backstrom et al., 2011). NS1 protein plays multiple roles during viral replication and is not incorporated in progeny virus (Hale et al., 2008; Shaw et al., 2008; Matsuda et al., 2010; Nivitchanyong et al., 2011). For example, NS1 interacts with phosphorylated serine threonine kinase Akt in cells and enhances Akt promoting anti-apoptotic activity (Matsuda et al., 2010). NS1also inhibits interferon production and antiviral effects subsequently induced by interferon, and enhances viral protein translation is related with NS1 (Hale et al., 2008). NS2 is also known as nuclear export protein (NEP) (Shaw et al., 2008). Both M1 and NS2 are involved in mediating export of RNPs from the nucleus (O'Neill et al., 1998; Akarsu et al., 2011). M2 tetramers in the

virus capsid serve as ion channels after virion entry into the endosome. Changes in endosomal pH cause a conformational change in HA which allows fusion with the endosomal membrane. In an independent event, M2 pumps protons into the virion which causes M1 (which is tightly associated with the RNPs) to release the RNPs so they can traffic to and enter the nucleus (Wang et al., 1993; Maclachlan and Dubovi, 2011). M2 has an extracellular domain (M2e) which has been considered a potential vaccine component (Neirynck et al., 1999). Since influenza is an enveloped virus, antibodies can only easily bind to protein domains spiking out of the virus membrane. HA, NA and M2 proteins all have extracellular domains outside of the virus membrane. These three proteins all have the potential to be vaccine targets to induce humoral responses.

Swine IAV disease

When pigs are infected with IAV, an acute disease in the respiratory tract is manifested similar to human infection. The incubation period of disease is 1 to 3 days followed by sudden disease onset of clinical signs and recovery. Recovery usually occurs within 7 to 10 days following infection (Vincent et al., 2008a). High morbidity and low mortality rates are observed by most swine IAV strains (Vincent et al., 2008a). Characterized clinical signs include fever, respiratory distress, coughing, sneezing, labored breathing, anorexia, and prostration (Richt et al., 2003; Ma et al. 2011). Purple to red multifocal or coalescing consolidated areas occur in cranio-ventral lung lobes are generally seen as gross lesions (Richt et al., 2003; Vincent et al., 2008a). Acute epithelial necrosis with subsequent attenuation or reactive proliferation, bronchointerstitial pneumonia, atelectasis, bronchiolitis, proteinaceous fluid and peribronchiolar lymphocytic infiltration are typical microscopic

changes within the lung (Richt et al., 2003; Vincent et al., 2008a; Ma et al., 2009; Ma et al., 2011). Virus shedding can be detected from nasal swabs and bronchoalveolar lavage (BAL) fluids. Young pigs are more susceptible to IAV than adult pigs (Richt et al., 2003). IAV co-infection with *Mycoplasma hyopneumoniae,* porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus type 2 (PCV2) and secondary bacterial infection in the respiratory tract of pigs is known as porcine respiratory disease complex (PRDC) (Thacker et al., 2001; Ellis et al., 2004; Fablet et al., 2011).

Swine IAV subtypes

IAV evolves continuously in two ways termed antigenic drift and antigenic shift. Minor changes of HA and NA proteins constitute antigenic drift including point mutations of nucleotides, i.e., substitutions, insertions and deletions (Murphy et al., 1999). Such minor mutations are due to polymerase errors which are common in RNA virus replication (Domingo et al., 1998; Gauger and Vincent, 2011). Antigenic drift may result in HA and NA types that are not recognized by antibodies induced prior to mutation. Antigenic shift constitutes major changes of gene combination or reassortment caused by exchange of whole gene segments between different strains which co-infect the same animal (Murphy et al., 1999; Vincent et al., 2008a). These two mechanisms of evolution give rise to the emergence of variant viruses. Pigs are susceptible to challenge of many subtypes of IAV (Kida et al., 1994). Before 1998, cH1N1 was the predominant subtype that caused most influenza infection in swine and had a predictable pattern similar to human influenza with prevalence in late fall and early winter (Easterday and Van Reeth, 2007). In 1998, an influenza outbreak in swine herds happened in several US states. The causative subtype was identified as H3N2

(Vincent et al., 2008a), a triple reassortant of gene segments from human-like H3N2 HA, NA, PB1 genes, avian-like PB2 and PA genes and cH1N1-like NS, NP, and M genes (Zhou et al., 1999; Webby et al., 2000). With time, triple reassortant H3N2 mutated and reassorted with cH1N1 to form new genotypes including new clusters of H3N2, H1N2 (HA from cH1N1 and other segments from H3N2), H3N1 and reassortant H1N1 (rH1N1, HA and NA from cH1N1 and other segments from H3N2) (Choi et al., 2002; Richt et al., 2003; Webby et al., 2004). Reassortment of H3N2 with HA and NA from human virus lineages H1N1 and H1N2 form huH1N1 and huH1N2 have been reported as spreading in US swine herds (Vincent et al., 2009; Lorusso et al., 2011). Within the H3N2 subtype, there are 4 phylogenetic clusters of H3N2 strain: I, II, III and IV (Richt et al., 2003; Hause et al., 2010; Olsen et al., 2006). Four phylogenetic clusters of swine H1 subtype have been identified in America: α (cH1N1), β (rH1N1), δ (huH1N1, huH1N2) and γ (H1N2), other than pandemic H1N1 (pH1N1) which forms clusters separated from North American viruses; cluster δ can be differentiated into two subclusters, $\delta 1$ (huH1N2) and $\delta 2$ (huH1N1) (Lorusso et al., 2011). MA and NA genes from Eurasian IAV reassorted with North American triple reassortant virus resulted in pandemic H1N1 (pH1N1), which caused disease in both humans and swine in 2009 (Garten et al., 2009; Moreno et al., 2010). Further reassortment of H3N2 and pH1N1 resulting in new IAV strains has been reported in Canadian swine in 2010 (Tremblay et al., 2011).

The main subtypes of IAV circulating in North American swine are H1N1, H1N2 and the H3N2 Cluster IV (Richt et al., 2003; Vincent et al., 2009; Kumar et al., 2011). According to the data from 2001 to 2007 of University of Minnesota Veterinary Diagnostic Laboratory,

in H1 subtypes, cluster α has been replaced with clusters β, δ and γ, and cluster IV became dominant in H3N2 subtypes (Rapp-Gabrielson et al., 2008). After the emergence of the pH1N1 subtype, the prevalence of pH1N1 in swine became significant. According to data from University of Minnesota Veterinary Diagnostic Laboratory, of all IAV isolates from swine in 2010, 27.8% were H3N2 cluster IV, 22.4% were pH1N1, 18% were huH1N2 δ1, 9% were huH1N1 δ2, 15.7% was H1N1 γ and the percentage of isolates belonging to α, β and other H3N2 clusters was less than 10% (Pfizer Inc., 2012). Influenza viruses of different clusters are antigenically divergent (Lorusso et al., 2011). Serologically, there is moderate to good HA antibody-antigen cross-reactivity between classical H1 and reassortment H1cluster β and H1cluster γ, but limited cross-reactivity among cluster β, γ and δ, or within δ cluster (Vincent et al., 2006; Vincent et al., 2008a; Vincent et al., 2009; Lorusso et al., 2011). For H3N2 IAV, cluster I and III, but not II, have HA antibody-antigen cross-reactivity between each cluster, and good reactivity within each cluster (Richt et al., 2003). The limited cross-reactivity of HA antibodies to HA antigens between different subtypes and clusters is one of the obstacles in developing vaccines to prevent all IAV viruses infecting swine.

Immune Responses of Influenza Infection in Swine

Influenza virus infection induces both cellular and humoral responses. Soluble effectors including cytokines are secreted as a part of the innate immune response to IAV infection. Pro-inflammatory cytokines, including interferon α (IFN-α), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1), are secreted in the lung associated with virus titers in pigs infected by IAV (Van Reeth, 2000). Cellular-mediated immune responses participate in

protecting swine from IAV infection. Proliferation of cross-reactive memory T-cells were detected in IAV recovered pigs that were free of maternal antibodies during infection, but not those with maternal antibodies present during infection (Kitikoon et al., 2006). IAV infected pigs with reactive memory T-cells recovered faster than those without such memory T-cells, so the presence of these cells in pigs may participate in rapid recovery from IAV infection (Kitikoon et al., 2006).

In adaptive humoral immunity, systemic and mucosal immune responses are induced following IAV infection, both of which are essential for the prevention and recovery from IAV infection (Cox et al., 2004). IAV specific IgG and IgA antibodies are believed to significantly contribute to virus clearance. Antibodies against HA are the most important in preventing infection, although, antibodies against NA contribute as well (Ma and Richt, 2010). Testing HI antibody titers in serum has been used widely to predict humoral immune response and protection against IAV infection because vaccinated pigs are protected from IAV infection by high HI antibody titers (Larsen et al., 2000;Vander Veen et al., 2009). Furthermore, testing HI titer in serum to different IAV virus subtypes can predict cross-protection efficacy. Because IAV targets mucosal cells in the respiratory tract, antibodies need to be transported to mucosal sites. Short-lived serum IgG antibodies and more durable local IgA antibodies occur in pigs recovering from influenza infection or when vaccinated via a mucosal route. These protected pigs from further IAV challenge (Charley et al., 2006). Virus specific IgG and IgA, as well as HI antibodies, peaked at 2 to 3 weeks after primary IAV infection in pigs. IgG is predominant in serum and IgA is predominant in the

respiratory tract (Larsen et al., 2000). IgA or IgG secreting cells in nasal mucosa are dramatically higher in numbers than that in any other tissues (Larsen et al., 2000).

Swine Influenza Vaccines

Vaccination can be an effective way to decrease IAV infection and reduce economic losses due to influenza pneumonia.

> *"There are three major problems with the control and prevention of SI in the U.S.: (a) SIV is changing faster than traditional vaccines can be developed, (b) There is a need for vaccines that can induce better crossprotection among SIV isolates, and (c) Passively acquired immunity is believed to block vaccine efficacy in pigs"* (Vincent et al., 2008a).

Similar point of view was indicated in another paper (Ma and Richt, 2010). Nevertheless, different types of IAV vaccine and strategies have been developed in order approach this aim (Table 1).

Inactivated virus vaccines

Commercial inactivated IAV vaccine for swine use became available in 1994 (Vincent et al., 2008a; Platt et al., 2011). Currently, inactivated whole virus IAV vaccine is the only commercially available vaccine used worldwide in swine herds (Ma and Richt, 2010). While inactivated whole virus IAV vaccines protect pigs fully against homologous IAV challenge, they only partially protect pigs against heterologous challenge (Vincent et al., 2010a; Vincent et al., 2010b).

To manufacture inactivated IAV, embryonated hen's eggs are generally used to propagate live virus (Gorres et al., 2011). Live virus is harvested from the eggs and killed with chemical treatment (Sanofi pasteur, 2009). Since frequent antigenic shift and drift of IAV occurs and older vaccines do not protect the infection of current circulating viruses, regulatory procedure for updating IAV strains in United States Department of Agriculture (USDA)-licensed veterinary vaccines is allowed to be expedited by USDA (Rapp-Gabrielson et al., 2008). It currently takes at least one year to update a commercial IAV vaccine (D.L. Harris, Iowa State University, personal communication). The approval of changing virus strains in vaccines is based on the efficacy and safety demonstration (Rapp-Gabrielson et al., 2008).

The most common vaccination route of influenza vaccines is intramuscular injection (IM). Intramuscular vaccination induces a high level of specific IgG antibody in serum and lung but lacks cross protection to other virus strains of different subtypes (Heinen et al., 2001). Immune responses of pigs naturally immunized by virus (A/Sw/Oedenrode/96 H3N2) infection or immunized by inactivated vaccine (A/Port Chalmers/1/73 H3N2, antigenic different from A/Sw/Oedenrode/96 H3N2) were compared post A/Sw/Oedenrode/96 challenge by Heinen et al. (2001). Results showed serum HI antibody titer, virus neutralizing antibody titer and nucleoprotein specific IgG antibody titer developed by vaccinated pigs were similar or higher than naturally immune pigs. However, vaccinated pigs developed lower nasal IgA titer and lower cell-mediated immune responses than naturally immune pigs (Heinen et al., 2001). Protection by this A/Port Chalmers/1/73 derived vaccine to A/Sw/Oedenrode/96 challenge was sub-optimal, because virus shedding was detected for a

short period in vaccinated pigs compared with no virus shedding from naturally immune pigs (Heinen et al., 2001). The result of this study indicated the limited cross-protection induced by inactivated IAV vaccine to heterologous challenge. This is the major problem of development of swine influenza vaccines. Vaccine strain mismatch with circulating strains may even enhance the severity of disease (Vincent et al., 2008b; Gauger et al., 2011). Therefore, HA antigenic match of vaccine strain and challenge strain is the key to providing protection by inactivated IAV vaccine. Commercial vaccines are often bivalent or trivalent, containing several circulating strains to increase chances of matching the challenge viruses. For example, Flusure XP (Pfizer, Inc., New York, NY USA) which is an IAV vaccine for swine use contains up to four swine influenza virus strains to provide cross protection (Lee et al., 2007; Pfizer Inc., 2011). However, it is difficult to cover all strains in a single dose of vaccine and manufacturing cost rises with increasing numbers of IAV strains in vaccines.

Another problem of IAV vaccination is the interference of maternal antibodies in piglets. Anti-influenza serum IgG is transferred to piglets from maternal antibodies in sow's colostrum. If sows are vaccinated with IAV vaccine before farrowing, positive IAV maternal antibodies (>40 fold in HI) can be detected in suckling piglets up to 14 weeks age (Markowska-Daniel et al., 2011). Swine influenza virus maternal antibodies are important for protecting young piglets and can cause of immunization failure (Wesley and Lager, 2006). Pigs vaccinated with commercial bivalent vaccine had better partial protection facing heterologous H1 challenge when maternal antibodies were absent than those possessing maternal antibodies (Kitikoon et al., 2006). It has been shown that the stimulation of IAV specific humoral responses and cellular responses in vaccinated pigs have both been

suppressed with the presence of maternal antibodies (Kitikoon et al., 2006).

Mucosal responses, including IgA antibodies and cellular responses, have been the focus of much study to attempt to provide wider cross-protection and override maternal antibodies. Intranasal (IN) administration of IAV vaccines has been attempted as an alternative method to protect pigs and induce local immune responses (Lim et al., 2001). Four doses in consecutive weeks of intranasal vaccination of inactivated influenza vaccine provided complete protection to pigs from homologous challenge, and IgG and IgA were detected in mucosal secretions and serum (Lim et al., 2001). IgG induction was detected after the 2nd vaccination, while IgA induction was detected following the 4th vaccination (Lim et al., 2001). The efficacy of mucosal administration of inactivated influenza vaccine to override the interference of maternal antibodies in piglets needs further evaluation. Four doses of vaccine are not convenient for practical vaccination on pig farms, and the cost of four doses may not be economically feasible. It would be helpful to determine the reason(s) that IgG induction requires fewer doses than mucosal IgA induction by inactivated vaccine in order to improve intranasal vaccine development.

Live attenuated vaccines

Recombinant modified influenza viruses can be obtained with reverse genetics technology and provides a novel way to make modified live attenuated virus vaccines (MLV). HA0 protein, the precursor of HA, must be cleaved into HA1 and HA2 in order to fuse with endosomal membranes (Skehel and Wiley, 2000). HA0 modified live attenuated virus (Δha0MLV) was achieved by introducing a mutation to the HA cleavage site (Stech et

al., 2005; Gabriel et al., 2008; Masic et al., 2010). Masic et al. (2009) showed that Δha0MLV could infect pigs without shedding live virus, proving that Δha0MLV is attenuated in pigs. The H1N1 strain of Δha0MLV administrated intranasally induced a significant cross protection to H1N1 and H3N2 challenge. Both macroscopic and histopathologic lung lesion scores were significantly reduced in both homologous challenge and heterologous challenge groups (Masic et al., 2010). Virus shedding was not detected in 5/6 pigs from homologous H1 strain challenge and not detected in 3/6 pigs from heterologous H3N2 challenge (Masic et al., 2010). Vaccinated pigs had considerable IgA in the lower respiratory tract and serum IgG after either homologous or heterologous challenge (Masic et al., 2010). Two dose vaccinations were required to induce protection for Δha0MLV (Masic et al., 2010).

NS1 of swine influenza virus has been demonstrated as a virulence factor with the function of antagonizing type I interferon (IFN-α/β) (Talon et al., 2000). Introducing mutations into the gene encoding the NS1 protein causes the loss of IFN-α/β inhibiting potency (Talon et al. 2000; Solorzano et al., 2005). NS1 modified influenza strain (Sw/TX/98Δ126) has been shown to be virulence attenuated in inoculated pigs by reducing lung lesion and live virus shedding(Solorzano et al., 2005). Induction of HI antibodies by this Δns1MLV indicated it was immunogenic (Solorzano et al., 2005). To test the efficacy of Δns1MLV as an influenza vaccine candidate, pigs were vaccinated intratracheally twice with Δns1MLV, followed by homologous or heterologous virus challenge. Homologous challenged pigs were completely protected by Δns1MLV and it was confirmed to be attenuated (Richt et al., 2006). In heterologous subtype challenge with a cH1N1 strain, macroscopic lung lesion scores in vaccinated pigs and unvaccinated pigs were similar, while

microscopic lung lesion scores and virus shedding in respiratory tract were reduced compared with the unvaccinated group (Richt et al., 2006).

In another study, two doses of intranasal vaccination of Δns1MLV attempted to evaluate heterologous protection efficacy. Attenuated live influenza virus with truncated NS1 (TX98 H3N2) was administrated intranasally (IN) by dripping vaccine into nares to evaluate homologous and heterologous protection. CO99 H3N2 (antigenic variant from TX98) and IA04 (H1N1) were the evaluated heterologous strains (Vincent et al., 2007). The results demonstrated that intranasal administration of Δns1MLV (TX98) significantly reduced rectal temperature, and live virus shedding and lung lesions following TX98 (cluster I) and CO99 (cluster II) challenge (Gramer et al., 2007; Vincent et al., 2007). Modest HI antibodies specific to TX98, but not CO99 or IA04 were detected in serum and robust IgA and IgG antibodies in mucosa with cross-reactivity to CO99 were detected in IN pigs (Vincent et al., 2007). The cross-reactive local antibodies induced by IN Δns1MLV vaccination indicate the potential of universal protection induction by intranasal vaccination. In addition, modest HI antibodies in serum indicate the potential to reduce IAV antibodies from sows to piglets through colostrum.

Modified live attenuated virus vaccine has not been approved for usage in pigs. Cold adapted MLV has been approved in the US for human (FluMist) and equine (FluAvert) use via the intranasal route (Belshe, 2004; Paillot et al., 2006). Cold adapted MLV is achieved by inducing combination mutations on genes coding PB1 and PB2 proteins of IAV, which causes virus replication to be temperature sensitive (Solorzano et al., 2005). FluMist vaccine strain is not only a cold adapted virus but also a reassorted virus strain. It contains 6 segments

(PB1, PB2, NP, M, PA and NS) from human H2N2 (A/Ann Arbor/6/60) that contribute to virus attenuation and 2 segments coding HA and NA protein from circulating wild type influenza virus (Chan et al., 2008).

Safety is a major concern for using attenuated live virus as a vaccine. Genome segments are able to reassort, there is concern that modified live vaccines will reassort with wild type strains in the field to produce novel virulent influenza strains. The reassortment of viral strain and vaccine strain require the replication of both of their genomes within a single cell at the same time. In all the vaccines studies above, live virus was not recovered from the upper respiratory tract after vaccination or in the lung. Whether there is live vaccine virus existing in the lung before challenge was not examined. Another concern of MLVs may revert to virulence over time if natural mutations occur (Babiuk et al., 2011).

Administration route is another problem for MLV in pigs. To induce local antibodies in respiratory mucosa, MLV normally requires intranasal administration. Commercial intranasal influenza vaccine for humans or horses is administrated in the form of mist. The narrow space of swine nasal cavity results in low efficiency of delivering mist deeply into the respiratory tract. Dripping vaccine into the nose, which is only commonly performed in an experimental setting, is time consuming and not likely to be efficient in a commercial farm setting.

DNA vaccines

Recombinant DNAs coding IAV proteins have been evaluated as vaccine candidates for swine. The era of gene vaccines was started by Wolff et al. in 1990 (Wolff et al., 1990).

They demonstrated that protein can be expressed upon direct inoculation of plasmid DNA into mouse muscle (Wolff et al., 1990). The advantages of DNA vaccination are (I) one recombinant DNA molecule can encode multiple genes of interest, which reduces manufacturing cost; (II) DNA vaccines do not carry infection risks associated with MLV vaccines; (III) recombinant DNA can express high levels of proteins of interest in cells; (IV) DNA vaccines have the capacity of inducing both humoral and cellular immune responses; (V) there is potential for DNA vaccines to override maternal antibodies which mainly recognize IAV surface proteins but not genomes (Dhama et al., 2008); (VI) capacity of differentiating infected from vaccinated animals (DIVA), because the DNA vaccine does not express all the proteins of the pathogens, and will induce different immune responses than naturally infected animals. The barrier of developing efficacious DNA vaccine is low DNA transportation efficiency into target cells using a traditional intramuscular (IM) vaccination route (Pertmer et al., 1995; Olsen, 2000; Dhama et al., 2008).

Gene gun delivery has been tested to enhance the efficiency of DNA transfection by Olsen and colleagues (2000). Two doses of gene gun administration of HA DNA vaccine to either pig skin or tongue resulted in high levels of HI antibodies after virus challenge; however, virus shedding was not completely prevented even with the co-administration of porcine interleukin-6 (Olsen, 2000; Larsen et al., 2001). This type of DNA vaccine immunization strategy may prime the pig immune system (Olsen, 2000; Larsen et al., 2001; Larsen and Olsen, 2002). Gene gun delivered DNA vaccine as prime and commercial vaccine as boost was found to reduce viral shedding similar to a two dose commercial vaccination strategy (Larsen et al., 2001).

Gorres et al. (2011) designed an IAV DNA vaccine by constructing a backbone with cytomegalovirus enhancer/promoter and the human T-cell leukemia virus type 1 R region recombined with trivalent HA genes (cH1N1, H3N2 and pH1N1) or monovalent HA gene (pH1N1) (Gorres et al., 2011). By three doses IM or needle-free (NF) 0.5-ml subcutaneous vaccination of pigs, similar levels of HI antibodies to vaccine strain virus and cellular immune responses were induced in both trivalent and monovalent groups with both IM and NF methods (Gorres et al., 2011). After H1 challenge, only IM and NF monovalent DNA vaccination reduced virus shedding at 3 days post challenge (dpc) and both NF trivalent and monovalent DNA vaccination completely protected against virus shedding at 5 dpc (Gorres et al., 2011). Post H3 challenge, both IM and NF Trivalent DNA vaccination reduced virus shedding at 3dpc and fully prevented virus shedding on 5 dpc. Minimal lung lesions were observed in examined vaccinated pigs (Gorres et al., 2011).

Nanoparticles like chitosan have been tested as IAV DNA vaccine adjuvants for sustained release of vaccine; however low transfection efficiency of the chitosan-DNA vaccine is a disadvantage of this approach (Zhao et al., 2011). There remains a need for identification of a more efficient method to deliver DNA vaccines in order to better evaluate this vaccination approach. So far, high dose of DNA vaccine is required for vaccination which is expensive and not practical.

Subunit IAV vaccines

A subunit vaccine is an immunizing agent containing viral proteins, but no viral nucleic acid (Myers, 2010). Subunit vaccines can contain higher concentrations of specific proteins than inactivated vaccines (Cox and Hollister, 2009). The major components of

subunit swine influenza vaccine is one or several recombinant IAV proteins, the viral structural proteins that are not composed in subunit vaccines or the antibodies against them can be detected to differentiate infection from vaccinated animals. Thus subunit vaccines have the potential to be DIVA. IAV structural protein HA is expressed in subunit influenza vaccines as it is able to induce HI antibodies (Cox and Hollister, 2009; Vander Veen et al., 2009; Shoji et al., 2011). Employing DNA recombinant technology, IAV proteins can be expressed in other platforms (Bachrach, 1982). Several platforms have been developed to express IAV subunits to replace traditional egg-based vaccine manufacturing.

A baculovirus expression system was used to produce influenza virus HA protein in insect cells as a vaccine to protect against influenza infection in humans (Cox and Hollister, 2009). Trivalent recombinant HAs, a combination of HAs derived from influenza A subtype H1N1, H3N2 and influenza B are comprised in a vaccine to achieve cross-protection (Cox and Hollister, 2009). In another method, HA, NA and M1 proteins of influenza virus expressed individually by baculovirus-insect platform self-assembled into high molecular-weight enveloped influenza virus-like particles (VLPs) (Bright et al., 2007). The capacity of such VLPs at inducing cellular and humoral immune responses has been demonstrated in preclinical trials (Bright et al., 2007). Nicotiana benthamiana was also developed as subunit expressing platform. H5N1 HA protein formed VLPs (H5 VLP) (D'Aoust et al., 2008). Cross-reactive antibodies were induced in a ferret model to influenza H5 VLP and a safety study in humans showed no significant induction of naturally occurring serum antibodies to plant-specific sugar moieties (Landry et al., 2010). Both of the above platforms are now

employed to produce experimental influenza subunit vaccine for human use, and may be candidates to produce vaccines for swine use.

An alphavirus replicon has also been employed to express IAV HA for swine vaccination (Vander Veen et al., 2009). There are two open reading frames (ORF) in the alphavirus genome (Rayner et al., 2002). All nonstructural proteins responsible for replicating viral RNA are encoded in the first ORF (Rayner et al., 2002). The second ORF of alphavirus normally encodes structural proteins which are responsible for the assembly of virus particles. An engineered virus genome, or replicon, containing the alphavirus ORF1 combined with heterologous genes in ORF2 electroporated into VERO cells and is able to express the heterologous proteins in high levels in cultured cells (Rayner et al., 2002). IAV HA protein expressed by alphavirus replicon platform in VERO cells was combined with adjuvant and tested as an IAV vaccine for swine (Vander Veen et al., 2009). This HA subunit IAV vaccine was efficacious in reducing virus shedding and gross and histopathologic lung lesions after homologous virus challenge and induced specific HI antibodies after pigs were vaccinated (Vander Veen et al., 2009). These subunit vaccines are made from alphavirus replicon are free of the possibility of replicating virus, since no structural genes of the alphavirus are present (Vander Veen et al., 2009).

Vectored vaccines

Vectors are utilized to transport genes into cells. Vectored vaccines containing genomic material can express high levels of the encoded protein after cell entry. Vectors may be replicative or replication defective. Replication defective vectors have the ability to infect cells but lack essential genes for production of new virus particles. Thus, vectored vaccines

need two essential features. The first one is that the vectors are able to infect cells and transport the recombinant genome into cells. The second essential feature is that the recombinant genomes are able to replicate by themselves and express the gene of interest in high levels in infected cells. Since the gene of interest in a vector is not all the genes of the target pathogen, vectored vaccine can be a DIVA vaccine. A wide host range of the virus vector and lack of pre-existing antibodies are two other important features to be considered for good vector candidates. Besides transporting the vaccine gene into cells, vector particles are able to stimulate cellular immunity to vaccine component, providing them an advantage over subunit protein vaccines (Macklin et al., 1998; Rayner et al., 2002; Wesley et al., 2004). Because they are expressed within cells, vectored vaccine antigens have the potential to avoid interference from maternal antibodies. Another advantage is that a vector can express several different genes (Vander Veen et al., 2012).

Human Adenovirus serotype 5 (Ad5) has been examined as an IAV vector for pigs (Wesley and Lager, 2006). Because the early transcription region 1 is deleted, Ad5 virus is replication-defective (Wesley et al., 2004). However, the recombinant gene of interest is able to be transcribed as it is under the control of constitutive promoters (Wesley et al., 2004). Pigs vaccinated with 1 dose mixture of Ad5 expressing the IAV H3N2 HA gene (Ad5 HA) and Ad5 expressing the H3N2 nucleoprotein (NP) gene (Ad5 NP) were completely protected from virus shedding and lung lesion development after homologous challenge. Pigs vaccinated with Ad5 HA shed low levels of virus and had low lung lesion scores which were not significantly different from that of Ad5 HA+Ad5 NP vaccinated pigs. Pigs vaccinated with Ad5 NP shed significant lower level of virus than unvaccinated pigs on 3 of 5 days after

challenge (Wesley et al., 2004). Furthermore, Ad5 expressing IAV H3N2 HA and NP proteins (Ad5-HA&NP) was shown to be able to prime the immune response in the presence of maternal IAV antibodies in piglets (Wesley and Lager, 2006). Piglets receiving maternal antibodies from gilts were administrated Ad5-HA&NP as a prime vaccine IM when suckling milk containing IAV maternal antibodies from gilts and boosted by commercial inactivated vaccine (End-FLUence 2, Intervet Inc., Millsboro, Del.) IM. Sows had antibodies to IAV H3N2 strain and End-FLUence 2 comprises H1N1 and H3N2 strains. Piglets were then challenged with a heterologous IAV H3N2 strain after boost vaccination. Better protection to these primed vaccinated piglets was induced compared with piglets without A5-HA&NP prime vaccinated pigs (Wesley and Lager, 2006). A disadvantage of Adenovirus vectors is the development of vector immunity (Pandey et al., 2012). Pigs can only be primed with adenovirus vectors, the efficacy of a boost injection will be inhibited by antibodies to the vector.

Alphavirus has also been developed as a vaccine vector based on an alphavirus replicon. Since genes coding structural proteins of alphavirus are deleted in the alphavirus replicon, new alphavirus particles cannot be produced. To make vaccine alphavirus particle vectors, alphavirus structural protein genes as helper RNAs are transported into cells *in trans* along with the replicon during electroporation. Alphavirus-like particles, known as replicon particles (RP) are formed subsequently (Rayner et al., 2002).

There are several advantages of alphavirus RP that make it an attractive vaccine platform candidate. Firstly, vector safety has been proven that RPs are not shed or spread to cohorts or into environment by vaccinated animals (Vander Veen et al., 2012b). Replication

deficient feature of RP was achieved by several techniques (1) dividing structural protein genes into two elements (Pushko et al., 1997; Smerdou and Liljestrom, 1999); (2) deleting 26S promoters and a big portion of non-coding sequences in front of 26S including starting codons and stop codons in each helper elements (K.I. Kamrud, Harrisvaccines Inc., personal communication). Secondly, anti-alphavirus vector immunity is minimal, thus the same individual animals can be multiply vaccinated with RP vector based vaccines. Thirdly, a rapid development of new vaccine is capable with RP system (Vander Veen et al., 2012a). Within 6 weeks, an IAV subunit or RP can be prepared with RP system, from receiving virus samples (R. L. Vander Veen, Harrisvaccines Inc., personal communication). Therefore, this advantage of RP system offers great potential for developing autogenous IAV vaccine. In addition, RP vector has dendritic cell tropism, and express multivalent genes of interest (Vander Veen et al., 2012a). Similar as other vectors, RP based vaccine has the capacity of DIVA, and able to express high levels of heterologous genes.

Venezeulan equine encephalitis virus (VEEV), a member of the alphavirus family, was selected as an IAV vaccine vector expressing HA protein. The VEEV has been shown to infect pigs, but only induces a transient viremia (Dickerman et al., 1973). Attenuated TC-83 VEEV strain, which is a biosafety level (BL) 2 pathogen has been developed as an RP vector (Erdman et al., 2010). This makes manufacturing of RP based vaccine easier and safer than using original BL3 pathogen-3014 VEEV strain. Pigs receiving two doses of RP vaccine expressing HA (HA RP) produced a high level of HI antibody from 7 days post boost vaccination and maintained this level at least 40 days (Erdman et al., 2010). HA RP encoding HA gene of cluster IV H3N2 or pH1N1 protecting pigs from homologous IAV challenge in

two other studies confirmed the efficacy of RP vaccine (Vander Veen, 2011; Vander Veen et al., 2012b). In pigs vaccinated with HA RPs, live virus was not isolated from nasal swabs and BAL samples and lung lesions were significantly reduced, in addition to HI antibodies, cell mediated immunity (CMI) was stimulated by HA RP (Vander Veen et al., 2012b). Mucosal antibodies specific to vaccine antigen was induced by vaccine adjuvanted with alphavirus RP in mice model, even the vaccine administration site was not mucosa (Thompson et al., 2008).

RP encoding homologous NP gene to challenge virus was shown to partially reduce virus replication in pigs and to stimulate both humoral antibodies and CMI, and the CMI responded to both homologous and heterologous IAV antigens *in vitro* (Vander Veen, 2011). Homologous NP RP itself did not protect pigs as well as HA RP against homologous challenge, NP RP along with HA RP has the potential to help piglets override maternal antibodies, which could be determined in a future study (Wesley and Lager, 2006; Vander Veen, 2011).

Pseudorabies virus (PRV), an alpha-herpesvirus in *herpesviridae*, has been used as a vaccine vector to express IAV HA as well. PRV consists a linear double-stranded DNA genome with the length of 14.5 kb. Several non-essential genes exist in PRV genome, most of which can be deleted to reduce virulence and replaced by other genes without affecting virus replication (Tian et al., 2006). Based on these features, a commercial attenuated PRV DIVA vaccine was developed with gE deletion (Pensaert et al., 2004). In addition, the attenuated PRV vaccine strain (Bartha-K61) was employed as an influenza vaccine vector expressing HA (Tian et al., 2006; Li et al., 2010). Recombinant PRV expressing H3N2 HA (rPRV-HA) protected mice against homologous virus challenge (Tian et al., 2006). In rPRV-

HA vaccinated group, live virus was not isolated from 4 dpc until the end of study from lung tissues and lung lesions were mild (Tian et al., 2006). Since this PRV vector is not suitable to be used multiple times due to immunity to the vector, prime/boost immunization with DNA expressing soluble HA fused with three copies murine complement C3d (HA/C3d DNA) and rPRV-HA was pursued (Li et al., 2010). This regimen induced better protection than one dose vaccination rPRV-HA in mice (Li et al., 2010). However, the protection to pigs provided by PRV vector influenza vaccine needs further evaluation. Attenuate PRV strain has good safety record and broad host range (Klupp et al., 2004; Yuan et al., 2008). These advantages further support PRV as an influenza vaccine vector candidate. However, immunity to vector limits the PRV vector use with multiple doses. Furthermore, the use of attenuated PRV strain as a vector may interfere with the surveillance of PRV vaccination (Ma and Richt, 2010).

Vaccinia virus, composing double stranded DNA genomes, has been developed to another influenza vaccine vector. Modified vaccinia Ankara (MVA) is an attenuated vaccinia strain used widely to eradicate human smallpox (Rimmelzwaan and Sutter, 2009). The attenuation of MVA is created by continual passages on primary chicken embryo fibroblast cells (Verheust et al., 2012). The immunity induced by recombinant MVA expressing IAV HAs was shown to be protective in equine and mice (Breathnach et al., 2006; Kreijtz et al., 2007). In addition, this vector can be used with the presence of pre-existing immunity in mice (Ramirez et al., 2000). These results indicate that MVA is competent as an influenza vaccine vector (Rimmelzwaan and Sutter, 2009), while whether MVAs expressing IAV proteins protect pigs need to be demonstrated.

Autogenous vaccines

Autogenous vaccines are prepared from field virus strains, and normally are inactivated virus. In general, it takes 8 to 12 weeks to produce an inactivated autogenous vaccine (R. L. Vander Veen, Harrisvaccines Inc., personal communication). The use of autogenous vaccines has increased in recent years because of rapid mutation rates of IAV and the difficulty of vaccine manufactures in updating their vaccine strains for commercial availability (Vincent et al., 2008a). An estimated $16.06 million in swine autogenous vaccines were sold in the US from May 1999 to April 2000 (Draayer, 2004). IAV and porcine reproductive and respiratory syndrome disease vaccines consisted of more than half of the autogenous vaccines produced (Draayer, 2004). In 2006, more than 20% of all known IAV vaccinated breeding sows and more than 9% of IAV vaccinated nursery-age pigs were vaccinated with autogenous IAV vaccines (USDA, 2007). Up to 2010, around 50% of IAV vaccines used for swine in US market were autogenous vaccines (Ma and Richt, 2010b). Autogenous vaccine production is normally achieved by inactivating live virus isolated from an infected herd and propagating the virus (Vincent et al., 2008a). Usage of such autogenous vaccines is only allowed in the herd from which the vaccine virus was extracted under a veterinarian's direction (Vincent et al., 2008a). Two to four different subtype viruses are typically contained within an autogenous vaccine mixture, but there can be up to 5 viruses. Efficacy of vaccine which would not be evaluated when vaccines were manufactured, but they may be monitored under a veterinarian's supervision at the time of use. Recently, the USDA Center for Veterinary Biologics (CVB) has notified vaccine manufacturers that recombinant non-living vaccines may be licensed as autogenous vaccines (USDA, 2012).

Conclusion

Current vaccine experiments have identified several alternative methods for safer and more rapid (ex. subunit vaccine, DNA vaccine or vector vaccine) vaccine production than traditional egg-based manufacture. Without expressing all proteins from the pathogen, these vaccines have the capability to be DIVA. However, most of these experimental vaccines, which are intended to protect infection from different influenza subtypes, has achieved optimal efficacy. IN MLV has nearly complete cross-protection to heterologous challenge and induced mucosal antibodies; however, safety is a concern and a practical IN method needs to be developed. As most subunit vaccine components are structural proteins of IAV, whether maternal antibodies will interfere with vaccine efficacy needs further evaluation. DNA vaccines may be an improved version of subunit vaccines as a result of high level expression of influenza protein(s) in hosts, although delivering DNA vaccine into cells erodes the efficiency of DNA vaccines. Vector vaccine may be regarded as superior to DNA vaccines, and is an alternative approach for DNA delivery into cells. HA protein(s) is the major antigenic component for induction of humoral HI antibodies through expression by these advanced vaccine approaches. Unfortunately, there remains the question of limited cross protection to heterologous challenges.

The current method to protect against diverse influenza virus strains for commercial inactivated vaccine is, to include various HAs in the same inactivated vaccine dose. An alternative way is the use of autogenous vaccine specific to the virus strain in certain swine herd, which can be prepared rapidly. However, a universal IAV vaccine protecting pigs is still desirable.

Table 1 Summary of characters of different types of influenza vaccines and vaccine candidates for Swine use

Vaccine types		Features	Preferred route of administration	Advantages	Disadvantages	Selected references
Inactiv-ated vaccin-es	Commercial	Inactivated whole virus	Intramuscular	Commercial available; good homologous protection by humoral antibodies	Limited cross-protection; mismatch of strains enhance severity of disease; maternal antibody interference; slow manufacture	Heinen et al., 2001; Kitikoon et al., 2006; Lim et al, 2001;
	Autogenous			Specific to circulating strains; Rapid updating	Virus source herd only; efficacy unknown until use	Vincent et al., 2008a
Modified live virus		Live whole virus, reduced virulence	Intranasal	Partial cross-protection; mucosal antibodies	Safety concern; impractical intranasal administration	Masic et al., 2010; Richt et al., 2006; Solorzano et al., 2005; Vincent et al., 2007
DNA		Nucleic acid only, without protein	Intradermal/Subcutaneous (gene gun or needleless injection)	Multivalent; non-infective; CMI responses; potential to override maternal antibodies; DIVA	High dose is required to provide sufficient protection	Gorres et al., 2011; Larsen et al., 2001

Continued of Table 1.

Subunit	Viral protein only, without nucleus acid	Intramuscular	Safe; pure target proteins; rapid manufacture; DIVA; possible to be available as autogenous vaccine	Potential to be interfered by maternal antibodies	Bright et al., 2007; Cox and Hollister, 2009; Vander Veen et al., 2009
Vector	Vectors containing vaccine genes, expressing vaccine proteins; Vectors: Adenovirus Alphavirus Pseudorabies virus Vaccinia virus	Intramuscular	Efficient transportation of GOI into host cell; multivalent; CMI responses; safe; (potential to) overriding maternal antibodies; rapid manufacture; DIVA	Immunity to some vectors;	Wesley and Lager, 2006; Bosworth et al., 2010; Erdman et al., 2010; Vander Veen et al., 2011; Vander Veen et al., 2012b

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CHAPTER 3. IMMUNIZATION FOR INFLUENZA A VIRUS BY INTRANASAL ADMINISTRATION OF ALPHAVIRUS REPLICON PARTICLES

Abstract

An influenza A virus (IAV) vaccine that provides better cross protection to pigs against heterologous strain infection and overrides maternal antibodies interference is needed. A replicon particle (RP) platform has been developed for influenza vaccine by expressing hemagglutinin (HA) protein (HA RP). Prime/boost intramuscular (IM) administration of HA RP is able to induce protective humoral antibodies to homologous challenge. In this study, we evaluated the efficacy of intranasal (IN) administration with HA RP of pandemic H1N1 (A/CA/04/2009 H1N1) in two pig experiments. In the first experiment, prime/boost RP vaccination was administered IN/IN to pigs, with IM/IM administration as positive control and sham vaccination as negative control (five pigs each group). All pigs were challenged by homologous challenge following two doses vaccination. Pigs were not protected by IN/IN administration of RP against IAV challenge. Virus titers and pneumonia were not reduced significantly in RP IN/IN administered group, compared with control group. In the second experiment, pigs were administered with one dose HA RP vaccine IM or IN, or with a combination of IN/IM routes with an interval of three weeks. In IN/IM or one dose IM administered pigs, At most two pigs at 2 DPC and 3 DPC, and no pigs at 3 DPC to 5DPC were isolated with live virus. All five pigs were detected with live virus from one dose RP IN administered pigs and sham vaccinated pigs. We also found that one

dose IM and combination of IN/IM vaccination with HA RP reduced pneumonia significantly compared with sham vaccinated group, contrary to one dose IN vaccination.

Introduction

Vaccination of pigs is a common method to control IAV infection in swine. Unlike the human influenza seasonal epidemiology, IAV infection of swine is not seasonal and there are many circulating variants in North America (Vincent et al., 2008). To protect variant influenza strains, the current strategy of commercial inactivated vaccines is making multivalent vaccines. However, the number of IAV variants circulating in North America are too numerous for a bivalent or trivalent inactivated vaccine to comprise (Vincent et al., 2008). Another major problem of vaccinating pigs with IAV vaccine is that maternal antibodies interfere with the vaccine efficacy. Therefore, an IAV vaccine, which provides better protection in pigs against heterologous strain infection and override maternal antibodies interference, is needed.

Replication deficient Alphavirus vectored IAV vaccines were developed as alternative vaccine candidates. The *Alphavirus* genus belongs to the family *Togaviridae* and contains a positive-sense, single-stranded RNA genome of approximately 11.5 kb in length (Griffin, 2007). Attenuated TC-83 Venezuelan equine encephalitis virus (VEEV) strain has been developed as a replication deficient vaccine vector, termed as replicon particles (RP), to express high levels of IAV hemagglutinin (HA) genes (Erdman et al., 2010). VEEV RPs also have a proven safety record demonstrating that RPs do not spread into the environment or to control pigs (Vander Veen et al., 2012). Therefore, RP based vaccine, with this safety

advantage, is a good commercial vaccine candidate. RP expressing HA proteins protected homologous challenge and induced robust humoral immune responses administered intramuscularly (IM) with prime/boost strategy (Vander Veen, 2011;Vander Veen et al., 2012).

Intranasal (IN) administration of inactivated or modified live virus (MLV) IAV vaccines has been demonstrated as an alternative administration route that protects pigs and induces local immune responses (Lim et al., 2001). Theoretically, inducing local immune responses to IAV infection has a potential to override the interference of humoral maternal antibodies. Another alternative vaccination strategy is administering pigs only one dose vaccine. An Adenovirus vector expressing HA protein was found to provide protection to pigs with only one injection vaccination at a high dose (Wesley et al., 2004). The combination of adenovirus vectored IAV vaccine (prime) and commercial inactivated IAV vaccine (boost) have been shown to override maternal antibodies successfully (Wesley and Lager, 2006). Efficacy of alternative vaccination strategies of RP in protecting pigs has not been evaluated.

Herein, we hypothesized that IN routes and combination of IN and IM routes of HA RP vaccination would protect pigs against homologous challenge, and these strategies had the potential to induce local antibodies and override the interference of maternal antibodies. In addition, we hypothesized one dose HA RP would be protective against homologous challenge. This study was composed of two animal experiments, the first study was to determine whether IN vaccination protect pigs against homologous challenge; the efficacy of

combination of IN and IM routes vaccination and one dose IM vaccination in protecting pigs against homologous challenge was evaluated in the second study.

Methods and Materials

Cells and virus

Madin-Darby Canine Kidney (MDCK) cells were passed in minimum essential medium (MEM) with 5% COSMIC Calf Serum, 1% Antibiotic-Antimycotic 100X (GIBCO® #15240, Gand Island, NY) and 1% MEM Non-Essential Amino Acids Solution 10 mM 100X (NEAA) in 5% CO_2 and 37 °C environment incubator. When cells were confluent in the flask, they were washed with phosphate buffered saline (PBS) and detached from the flask by trypsin 250 (Difco™ #215240, Houston, TX) incubation. Cells were split to new flasks with 1 to 3 area ratio.

Pandemic H1N1 (A/CA/04/2009) virus was propagated and titrated on MDCK cells as previously described by with modification (Szretter et al., 2006). MDCK cells were inoculated with 1ml pH1N1 of 10^5 $TCID_{50}$/ml and inoculation medium [MEM medium with 1% Antibiotic-Antimycotic 100X (GIBCO® #15240) and 2ug/ml TPCK Trypsin (Thermo Fisher Scientific Inc. #20233, Rockford, IL)]. Virus was filtered through a 0.45μm filter and stored at -80 °C before use. Virus was titrated on MDCK cells in 96 well plates by 10 fold serial dilution. Plates were incubated with inoculation medium in 5% CO_2 and 37 °C until cytopathic effect (CPE) did not showed up in any more wells. Infected cells were visualized by IFA similar to VERO cells as previously described (Vander Veen, 2011). Virus

concentration was calculated by 50% Tissue Culture Infectious Dose ($TCID_{50}$/ml) as described previously (Reed and Muench, 1938).

Replicon particle vaccine

Alphavirus replicon particles, expressing HA protein (HA RP) of pH1N1, was prepared as previously described (Kamrud et al., 2007). Briefly, HA gene cDNA was amplified by reverse-transcript polymerase-chain reaction (RT-PCR), using primers that contained EcoRV and AscI restriction sites were added into the 5' and 3' end of the HA cDNA during amplification. HA cDNA was then cloned into a replicon plasmid which contained non-structural protein genes of alphavirus (α-nsps) using these two restriction sites. This plasmid was amplified in *Escherichia coli* (*E.coli*) to a large amount. Replicon RNA, comprised of α-nsps/HA genes was transcribed from the plasmid *in vitro* and then purified. Helper RNAs were transcribed from helper plasmids with similar method to replicon. Replicon and helper RNAs were electroporated *in trans* into Vero cells in electroporation chambers. Electroporated Vero cells were incubated at 37 °C with the same medium as MDCK growth medium and 5% CO_2 for 18 hours. HA RP was harvested from cell culture. Infectious unit concentration of RP was titrated on Vero cells with indirect immunofluorescence assay (IFA) (Kamrud et al. 2007). Goat anti-VEE NSP2 antibody was used as the primary antibody and Alexa Flour® 488 donkey anti-goat IgG (H+L) (Invitrogen™ #A11055, Grand Island, NY) was used as the secondary antibody. Vero cells infected with RP were observed microscopically at 488nm wave length filter.

Animal studies

Experiment 1

Three week old commercial pigs were used in this study. Fifteen pigs were confirmed negative for IAV and porcine reproductive and respiratory syndrome virus (PRRSV) antibodies by commercial ELISAs against IAV NP protein (IDEXX AI multiS-Screen Ab Test by IDEXX Laboratories, Westbrook, ME) and PRRSV NP protein (HerdChek X3 by IDEXX Laboratories, Westbrook, ME). ELISA assays were performed by Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). Pigs were randomly sorted by weight into three groups with five pigs per group and assigned to three separated rooms at arrival. Ceftiofur per labeled dose antibiotic (Excede® for swine by Pfizer Animal Health, New York, NY) was injected intramuscularly to prevent bacterial infection. At four week of age (day 0), Control pigs received 2ml PBS intra-nasally as placebo, while the other two groups were administered 2ml pH1N1 HA RP containing 10^8 IU RP intra-nasally (IN/IN group) with an aerosolization instrument (Pump It by Genesis Instruments Inc., Elmwood, WI) or intramuscularly (IM/IM group) (Table 1). On day 21, pigs were administered the same vaccine or placebo according to their group as a booster vaccination. On day 42, all pigs were challenged intratracheally with 10 ml IAV pH1N1 strain virus at a dose of 10^4 $TCID_{50}$/ml. On day 47, all pigs were euthanized and necropsied. Pigs were sedated with IM Telazol® (100mg/ml, Fort Dodge Animal Health, Fort Dodge, IA) at a dosage of 1ml per 100 pounds IM. Beuthaniasia-D (Schering-Plough Animal Health, Union, NJ) with a dosage of 1ml per 10 pounds given intravenously (IV) was used for euthanasia. The Institutional

Animal Care and Use Committee (IACUC) at Iowa State University approved this animal study.

Experiment 2

Twenty-five, three week old commercial weaned pigs were randomized by weight into five groups with five pigs in each group upon arrival and assigned to one of five rooms isolated from each other. Serum samples were collected and evaluated by commercial ELISAs against influenza A NP protein (IDEXX AI multiS-Screen Ab Test by IDEXX Laboratories) and PRRSV NP protein (HerdChek X3 by IDEXX Laboratories). ELISA confirmed that all serum samples were negative of SIV and PRRSV antibodies. Antibiotic per label dose was injected intramuscularly. The pigs were then treated with different vaccination protocols as outlined in Table 2. The (IM)/(IM) group served as negative vaccination control. All HA RP doses were 10^8 IU in 2ml. All pigs were challenged intratracheally with pH1N1 strain of IAV at dose of 10^4 $TCID_{50}$/ml by10 ml on day 42. On day 47, all pigs were euthanized and necropsied as experiment 1. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University.

Sample collection

For both experiments, blood samples were collected before prime vaccination (day 0) and once a week after day 0, before challenge (day 42), and at necropsy (day 47). Nasal swabs were collected daily from day 41 until necropsy. Nasal swab samples were kept in 1.5ml MEM medium with 2% Antibiotic-Antimycotic. Bronchoalvoelar lavage (BAL)

samples were collected during necropsy. All blood, nasal swab and BAL samples were transported in ice and stored at -80 °C until use. At necropsy, lungs were removed and tissue samples from each lobe and trachea were collected and fixed in 10% formalin.

Clinical observation

Coughing, respiratory rate and behavior, were observed daily from one day prior challenge. Coughing was scored between 1 and 4 to reflect the severity according to the following criteria: 1= none; 2= soft or mild intermittent cough; 3= harsh or severe, repetitive cough; 4= dead. Respiration were scored between 1 and 4 to reflect respiratory syndrome severity according to the following criteria: 1= normal; 2= panting/rapid; 3= dyspnea; 4= dead. Behavior was assigned a score between 1 and 4 according to the following criteria: 1= normal; 2= mild lethargy; 3= severe lethargy or recumbancy; 4= dead. Coughing score, respiration score and behavior score of each pig on every observed day were summed up as a clinical score. Body temperatures were determined twice a day from sub-dermal chips in the morning and evening from one day prior challenge until the end of animal study. Electronic temperature measuring chips (LifeChip® by Destron Fearing™, South St. Paul, MN) were injected intramuscularly into pigs' necks. Pigs' body temperatures could be received by Bluetooth scanner, when the scanner was close to pig's neck. Weight was determined at arrival, before challenge and at necropsy. Daily weight gain (Lb) after challenge was calculated.

Live virus isolation

Live virus titers were determined with medium containing nasal swab samples and BAL samples as previously described with modification (Vincent et al. 2010). When MDCK cells were at monolayer on 96-well cell culture plates, supernatant was removed from the plates. Medium submerging nasal swab and BAL were thawed and centrifuged to discard cellular debris. Supernatant were diluted 10-fold from 10^{-1} to 10^{-7} in another 96-plate. Diluent was MEM medium containing 1% Antibiotic-Antimycotic (GIBCO® #15240) and 0.2mg/ml TPCK trypsin (Thermo Fisher Scientific Inc. #20233). Diluted samples (100 μl) from each well were transferred to MDCK plates. MDCK plates were incubated in 37 °C with 5% CO_2 for 3-5 days until no CPE showed up in any more wells. Titers were calculated as $TCID_{50}$/ml as virus isolation protocol.

Hemaglutination inhibition assay

Antibodies in pig sera against influenza HA protein, were measured by HI assay performed by ISU VDL following standard laboratory protocol. Briefly, serum samples from pigs were treated with receptor-destroying enzyme overnight to remove nonspecific hemagglutinin inhibitors in serum. Complements in serum samples were heat inactivated. The serum samples were absorbed with 20% turkey erythrocytes and centrifuged. Supernatants were serially diluted in u-shaped bottom well microtiter plates with an equal volume containing 4-8 agglutinating units of pH1N1 strain and plates were incubated at room temperature before addition of 0.5% turkey erythrocytes. Titer was defined as reciprocal of the maximal dilution that inhibited hemagglutination.

Pathology

The severity of pneumonia was determined by macro-pathological lung lesion scores and histopathological [hematoxylin and eosin (HE) and immunohistochemistry (IHC)] scores. Gross lung lesions were observed and assigned to a score from 0 to 100 based on weighted proportions reflecting the estimated total percentage of lesions to whole lung volume (Halbur et al., 1995). Fixed tissues were sent to ISU VDL for HE staining and Influenza virus A specific IHC staining (Vincent et al., 1996). HE stained samples were scored 0 to 3 reflecting the severity of bronchial epithelial injury (Richt et al., 2003; Vincent et al., 2007). IHC stained samples were scored 0 to 3 depending on amount of influenza virus antibody stained cells with the following criteria: 0= no staining; 1= mild staining; 2= moderate staining; 3= marked staining. All scoring work was done by a board-certified veterinary pathologist who was blind to pig treatment.

Statistical analysis

Single factor analysis of variance (ANOVA) was used to analyze temperatures, average daily gain of weights, gross lung lesion scores, histopathological lung lesion scores, log 10 transformed nasal swab and BAL viral titers. Statistical significance was set at two sides $p<0.05$.

Results

Experiment 1

Clinical evaluation

After challenge, clinical signs of influenza observed in this study were mild even in the sham vaccinated group. At most, two coughing pigs were observed in each group every day. No respiratory syndrome or lethargy was observed in any group after challenge until the end of study. Daily weight gain of sham group was 1.5±0.2, IN/IN group was 1.9±0.2 and IM/IM group was 1.4±0.2. The means of clinical scores of each day, means of body temperatures of each observation and means of AGD post challenge of IN/IN group and IM/IM group are compared with sham group, and no significant differences were observed.

Live virus isolation

Average titers of live virus isolation from nasal swab and BAL samples within each group, are summarized in Figure 1A. The number of pigs from which live virus was isolated were indicated as well. No live virus shedding was detected in IM/IM group post challenge in nasal swab samples or BAL samples. Live virus shedding was detected in nasal swab samples of both sham group and IN/IN group from 2 DPC until day of necropsy and in BAL samples. Means of virus titers of both groups were not significantly different. Means of virus titers from nasal swab samples peaked and all samples were isolated with live virus on 4 DPC in both groups. However, there were fewer pigs isolated with virus in IN/IN group than the sham group from nasal samples on 3 DPC (3 pigs in sham group 1 pig in IN/IN group)

and 5 DPC (4 pigs in sham group and 2 pigs in IN/IN group) and from BAL samples on 5DPC (4 pigs in sham group and 1 pig in IN/IN group).

Hemagglutin inhibition assay

HI assays to the challenge virus strain were performed and the Geometrical group means of HI titers are summarized in Figure 1B. On the day of boosting vaccination (day 21), mean of IM/IM group HI titers was 20, and was un-measurable in IN/IN group or sham group. Eight days later, mean of HI titers in IM/IM group increased to 140 and remained above this titer until the end of study, with the highest mean (243) on day 35. IN/IN group did not have a detectable HI titer before challenge (day 42) and HI antibodies with mean titer of 23 were detected after challenge (day 45). No detectable HI antibodies appeared in the sham vaccinated group throughout the study.

Macro-pathological and histopathological evaluation

Macro-pathological lung lesions, associated with influenza virus, were prominent in the sham vaccinated group. Macro-pathological lesions, including red consolidation mainly on cranioventral lung lobes, were observed in all pigs in sham (16.6±3.0) and IN/IN group (14±3.4) and two pigs in IM/IM group (1.6±1.0; Figure 2A). Necrosis of bronchial epithelial cells, inflammation around bronchia and neutrophil aggregation were observed on all HE stained specimens of sham (1.7±0.1) and IN/IN (1.8±0.3) groups and one of IM/IM group (0.2±0.2; Figure 2B). Influenza A, stained by IHC and showing brown color, was observed in bronchia epithelial cells in all sham (2±0.5) and IN/IN (1.4±0.2) groups samples, while was not observed in IM/IM group (Figure 2C). Mean macro-pathological scores and mean

histopathological scores (HE and IHC) of IM/IM group were significantly lower than the sham and IN/IN groups (Figure 2A,B&C). However, sham and IN/ IN groups did not have significant differences neither in macro-pathological scores, nor in histopathological scores.

Experiment 2

Clinical evaluation

Panting, rapid breathing and dyspnea, were observed post challenge in all groups, but these syndromes appeared prior to challenge. Behavior and appetite were normal post challenge in all groups. No lethargy was observed in any group. Means of clinical scores had no significant differences among all groups on any observed day. Compared with (IM)/(IM) group, a significantly higher mean body temperature was observed in IM/IM group at 36 hours prior challenge and significantly lower in IN/IM group at 60 hours post challenge. Other than these two significant differences, mean body temperatures for each group were not significantly different from (IM)/(IM) group. For Daily weight gain post challenge, IN/IM group (0.5±0.1) was significant lower than all the other groups. The other four groups, (IM)/(IM) group (1.2±0.1), IN/(IM) group (1.1±0.2), (IN)/IM group (1±0.3) and IM/IM group (1.2±0.1), had no significant difference.

Virus isolation

Virus shedding was completely eliminated from nasal swab or BAL samples in IM/IM group (Figure 3A). Live virus was detected in all pigs of IN/(IM) group and (IM)/(IM) group from nasal swab samples from 3 DPC to day of necropsy. BAL samples from three pigs in IN/(IM) group and four pigs in (IM)/(IM) group were detected with virus.

In IN/IM group and (IN)/IM group, live virus has been isolated at 2DPC and 3DPC, from at most two pigs from each group. Mean virus titers were both significantly lower than that of (IM)/(IM) group at 3DPC. Virus was cleared in these two groups from 4 DPC in nasal swab or BAL samples. There was no significant difference in virus titers detected between these two groups.

Hemagglutinin inhibition assay

After prime vaccination (day 0) in IM/IM group, there was less than 20-fold increase in group mean of HI antibodies, which lasted until the boost vaccination (day 21, Figure 3B). HI mean antibody titer increased to 139 (range from 80 to 320) after boost vaccination in IM/IM group and remained at least 80 until the end of the study. HI levels were not raised post challenge in IM/IM group. HI antibody titers of IN/IM group and (IN)/IM group showed a similar trend and there was no detectable HI antibody induced until two weeks post boost vaccination (day 35). On day 35, HI antibodies, of about 20-fold increase (range from 15 to 23), were detected and lasted until challenge in these two groups. Post challenge, HI antibodies increased to 70-fold (40 to 160) in both these groups. In IN/(IM) group and (IM)/(IM) group, no detectable HI antibodies were observed through out the study.

Macro-pathological and histopathological evaluation

Severe macro-pathological and histopathological lung lesions were observed in IN/(IM) group and (IM)/(IM) group. In acutely infected cells, influenza viruses stained by IHC were observed. In later infection, inflammatory cells gathered in the airway under epithelial cells and some epithelial cells were degenerated and neutrophils migrated in to the

airway. Pneumonia of IN/(IM) group was reduced when compared with (IM)/(IM) group, but this reduction was not significant (Figure 4 A,B&C). Pneumonia of the IM/IM group, IN/IM group and (IN)/IM group was significantly reduced compared with (IM)/(IM) group (Figure 4 A,B&C).

Discussion

In both experiments, two doses of IM vaccination induced a high level of HI antibodies post boost, completely eliminated homologous virus propagation in lung and nasal cavity, and significantly reduced lung lesions. These results confirm the efficacy of RP expressing pH1N1 HA protein as demonstrated in previous studies (Vander Veen, 2011).

Virus replication was partially inhibited by two doses of IN vaccination with HA RP. However, Virus titers of the IN/IN group were not reduced significantly when compared with the control group on any day, even fewer pigs had live virus isolated from nasal cavity on 3DPC and 5DPC and from lung on 5DPC. The shortened period of virus shedding did not limit the severity of pneumonia, however, it has the potential to limit virus spread within the herd and accelerate recovery from pneumonia. One dose IN vaccination did not prevent virus shedding by titer or pig numbers, pneumonia, nor prime humoral immune responses.

There were no detectable HI antibodies found in IN/IN group prior to challenge. Since the vaccine was administered to a mucosal site, the vaccine may not induce strong systemic immune responses. In contrast to the control group, there were low levels of HI antibodies induced in IN/IN group post challenge. This result agreed with a previous study, where pigs were protected by IN MLV, modest HI antibodies were induced in vaccinated

pigs, while local IgG and IgA levels in lung were significantly higher than that of unvaccinated pigs (Vincent et al., 2007). Compared with IM/IM administration, IN/IN administered HA RP did not provide protection to pigs against IAV challenge. A possible reason may be due to the inefficient administering instrument. The quantitative aerosolization instrument used in this study provided a fast and easy way to administer the vaccine intra-nasally. However, the vaccine droplets, that may not deeply penetrate the pigs' upper respiratory tract, may be sneezed out easily preventing vaccine adherence to respiratory epithelial cells. It is additionally possible that the numbers of RP infected cells was not sufficient to induce protective immune responses in this study.

Prior to challenge, HI titer in (IN)/IM group was about 20 fold, which was relatively low compared with IM/IM group. Three pigs in the (IN)/IM group were completely protected from virus shedding post challenge (data not shown). For pigs shedding virus in (IN)/IM group, the shedding was stopped faster than (IM)/(IM) group. This indicated that a low titer (lower than 20 fold) of HI antibodies could protect pigs facing influenza infection. In another study, when pigs were challenged with IAV, virus shedding was cleared at 7 days post challenge, while HI antibodies did not rise dramatically until 14 days post challenge (Larsen et al., 2000). This indicated that the clearance of virus may not require a high level of HI antibodies, which supported the result of current study. With the presence of such low level of HI antibodies circulating in serum, HI titer rose rapidly (in 7 days) after boost vaccination or after virus challenge, which resulted in the reduction of infection (Figure 4). Gross lung lesions in IM/IM, IN/IM and (IN)/IM groups were reduced significantly compared with (IM)/(IM) and IN/(IM) groups. No significant difference was found among IM/IM, IN/IM

and (IN)/IM groups. Therefore, the importance of low level of HI (<40 fold) was highlighted and one dose IM vaccination with RP was efficient to protect against influenza in pigs.

IN/IM vaccination protected against macro-pneumonia and micro-pneumonia better than (IN)/IM vaccination, although the differences were not statistically significant. Also, IN/IM vaccination partially protected against virus shedding and cleared up virus shedding as fast as (IN)/IM vaccination. This demonstrates protection induced by IN/IM administering HA RP against homologous challenge. Whether IN/IM vaccination provides better cross protection or is able to avoid interference by maternal antibodies is worth to be evaluated in future studies.

The challenge virus was delivered intratracheally, at a site lower than intra-nasal vaccination site. Whether the challenge site was protected by local antibodies induced by vaccine may be a concern. However, this should not be a factor that affects intranasal vaccination efficacy. MLV administered both intra-nasally or intratracheally protected homologous intratracheal virus challenge (Richt et al., 2006; Vincent et al., 2007).

There were pleurisies and fibrinouspleurisy in lungs observed in several pigs, which indicated chronic bacterial infection, and pigs showed clinical respiratory syndromes prior to challenge occasionally in experiment 2. This chronic pre-infection may have confused clinical syndromes caused by influenza infection post challenge. In addition, this might also affect pig growth. Three pigs in IN/IM group showed clinical syndromes prior to challenge. Growth of IN/IM group was significant slower than IN/(IM) group, IM/IM group and (IM)/(IM) group. The chronic pre-infection might contribute to the slower grown rate in IN/IM group. On the other hand, the 5 days period may not be long enough to show a

statistically significant difference in weight gain, and further, the sample size limited statistical analysis.

In conclusion, two doses of IM vaccination of pH1N1 HA RP protected against virus shedding completely and reduced pneumonia significantly in homologous challenge; two doses IN administration of HA RP did not protect pigs against IAV; one dose IM and combination IN/IM routes vaccination with HA RP reduced pneumonia significantly and partially inhibited virus shedding following homologous challenge.

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Table 2. Experiment 1: Study protocol.

Group name	Pig numbers	Day 0 prime vaccination	Day 21 boost vaccination	Day 42 challenge	Day 47 necropsy pigs
Sham	5	IM PBS	IM PBS	pH1N1 IAV[2]	5
IN/IN	5	IN RP[1]	IN RP	pH1N1 IAV	5
IM/IM	5	IM RP	IM RP	pH1N1 IAV	5

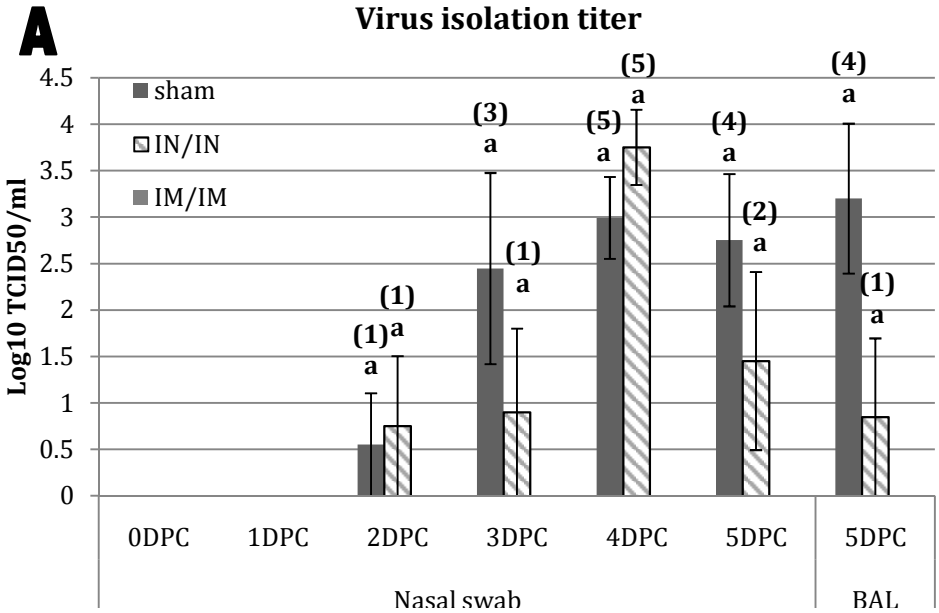
1: RP expressing HA protein of pandemic H1N1 subtype. 2: All challenged virus was in a dose of 10^4 $TCID_{50}$/ml in 10ml.

Table 2. Experiment 2: Treatment protocol.

Group name	Pig numbers	Day 0 prime vaccination	Day 21 boost vaccination	Day 42 challenge	Day 47 necropsy pigs
IN/(IM)	5	IN RP[1]	IM placebo	pH1N1 IAV[2]	5
IM/IM	5	IM RP	IM RP	pH1N1 IAV	5
IN/IM	5	IN RP	IM RP	pH1N1 IAV	5
(IN)/IM	5	IN placebo	IM RP	pH1N1 IAV	5
(IM)/(IM)	5	IM placebo	IM placebo	pH1N1 IAV	5

[1] RP expressing HA protein of pandemic H1N1 subtype. [2] All challenged virus was in a dose of 10^4 $TCID_{50}$/ml in 10ml.

Figure 1. Experiment 1. (A) Titers of live virus isolated from nasal swab samples and BAL samples from 0DPC until 5DPC, amount above each bar represents the number of pigs that live virus was isolated from, each group had 5 pigs until the end of study. (B) Means of HI titers to challenge virus (pH1N1) of each group, boosting vaccination was on 21 days post prime vaccination, pigs were challenged on day 42 and necropsied on day 47.



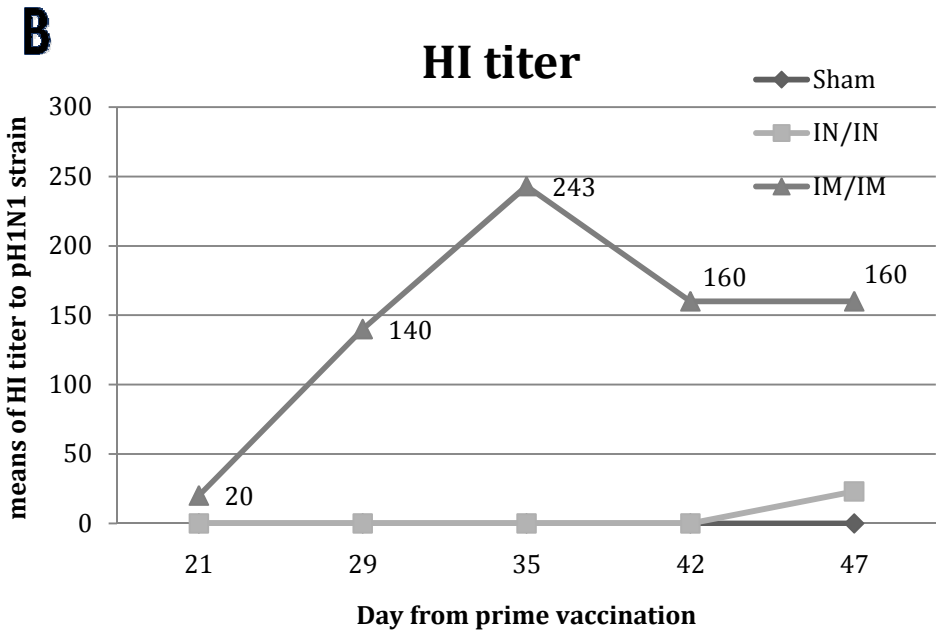


Figure 2. Experiment 1, volumes representing mean of lung scores with standard errors and same lowercase character mean no statistically significant difference. There is no significant difference between sham and IN/IN group, but IM/IM group was significantly lower than sham and IN/IN group, in macro-pathological scores, histopathological scores (HE and IHC). (A) Mean of macro-pathological lung lesion scores of each group; (B) histopathological lung lesion score means (HE); (C) Means of IHC staining scores of each groups.

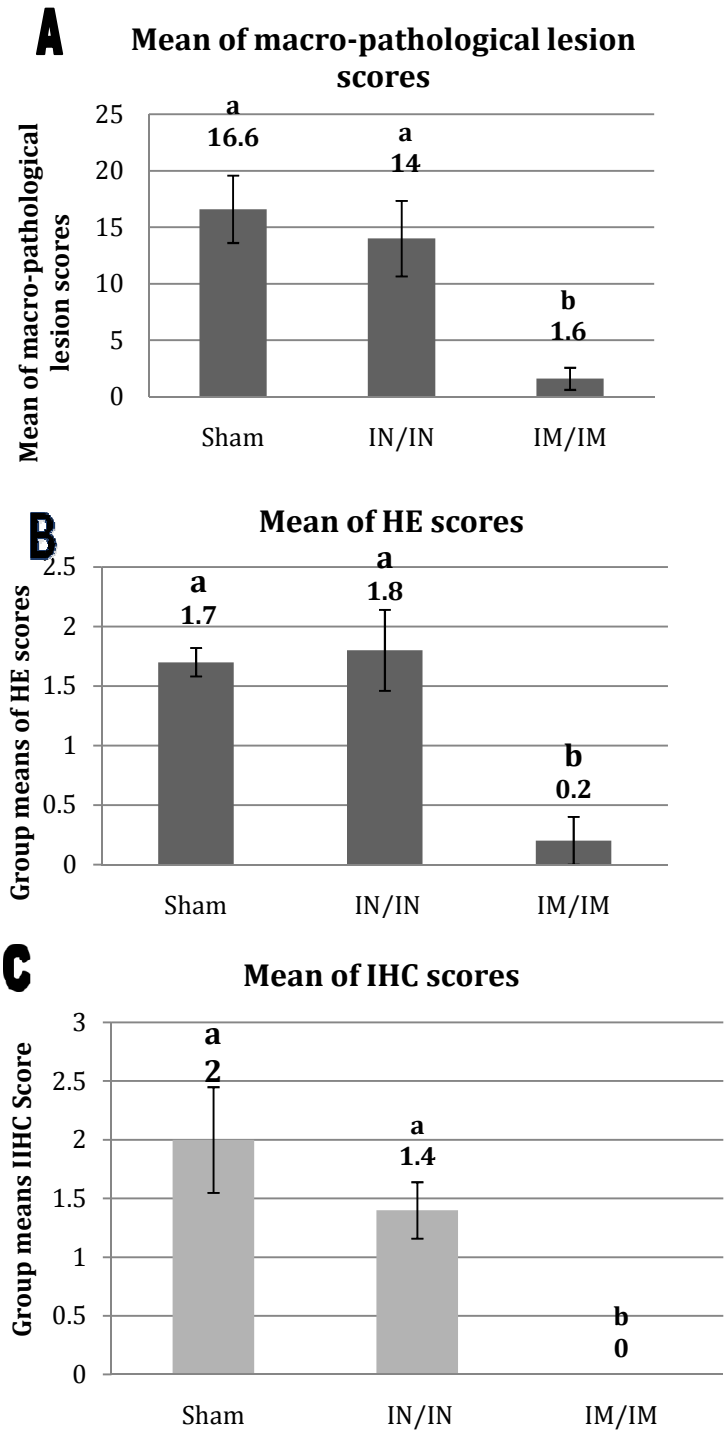
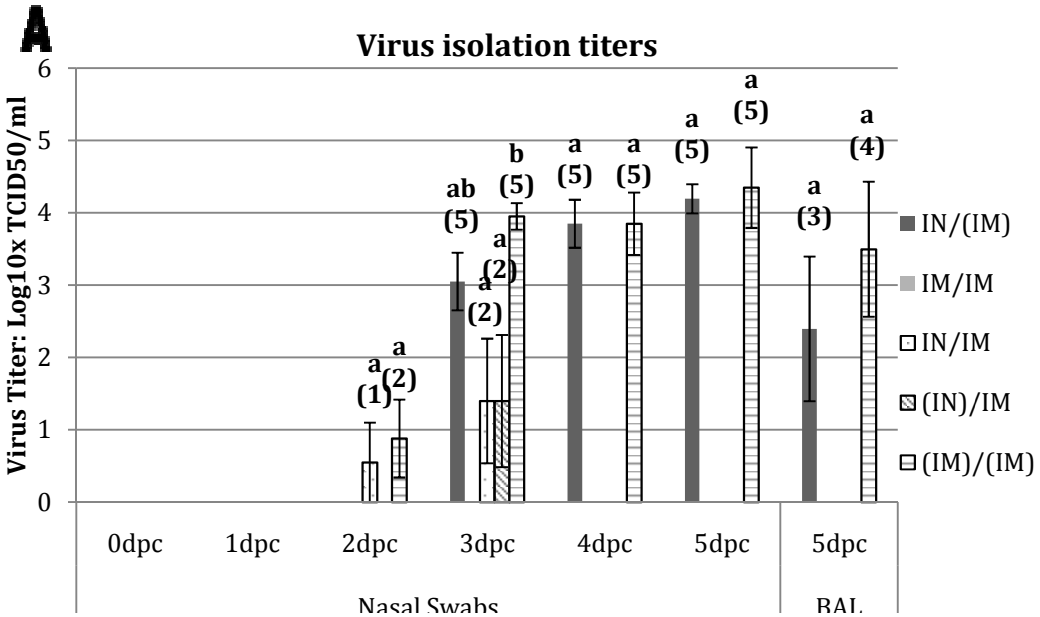


Figure 3. Experiment 2. (A) Volumes present means of virus titers with standard error bars, statistic analysis was evaluated between positive groups and presented by characters, numbers in brackets are the number of observed pigs shedding virus; (B) means of HI titers to homologous antigen of each group, boosting vaccination was on 21 days post prime vaccination (day 0), pigs were challenged on day 42 and necropsied on day 47.



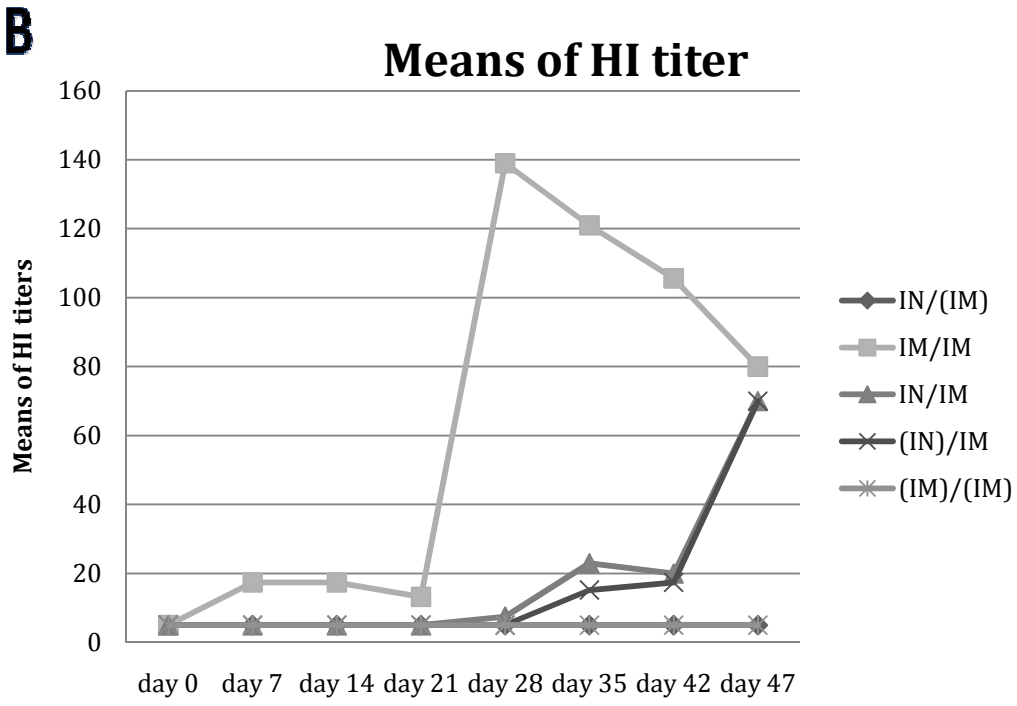
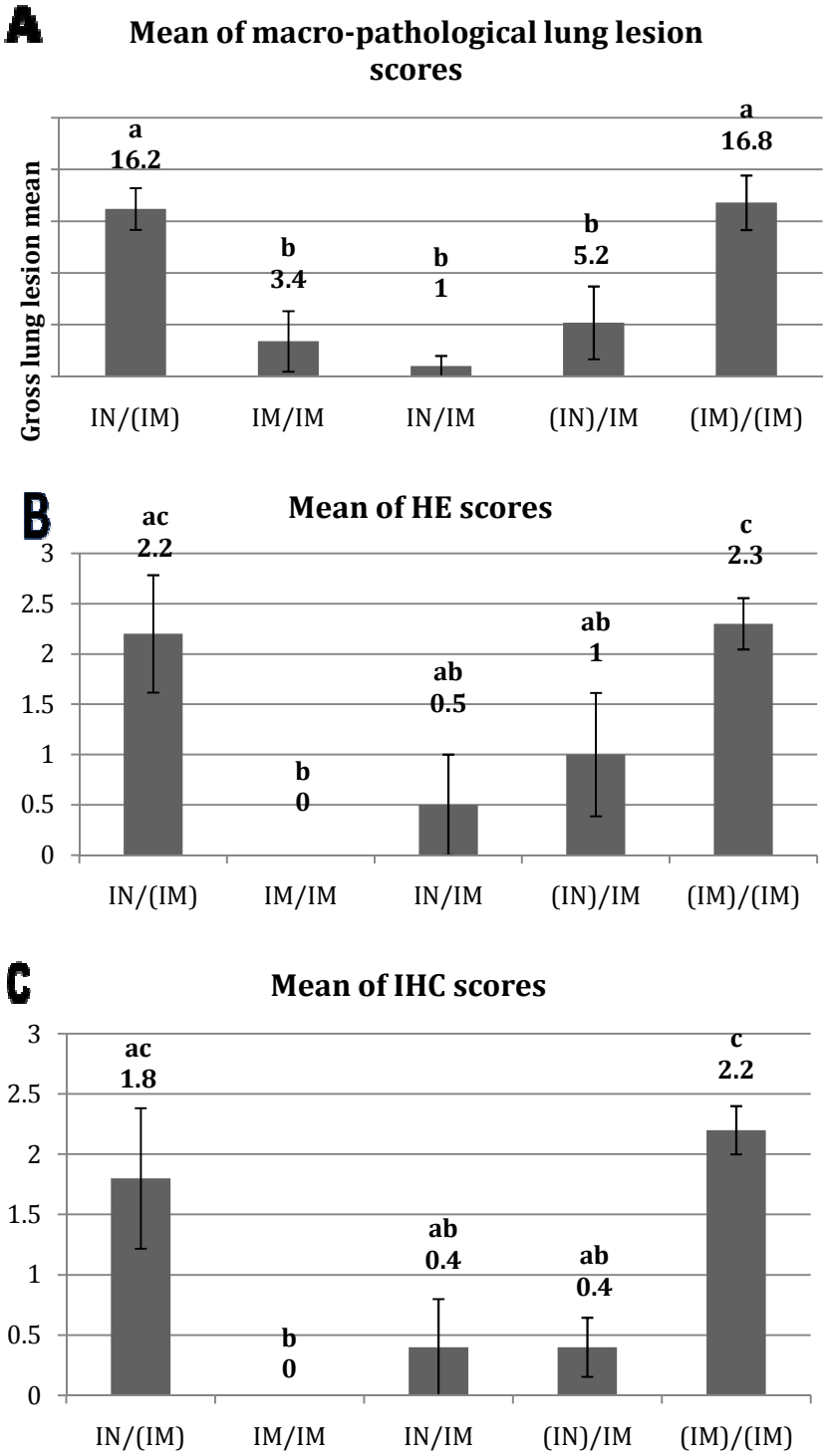


Figure 4. Experiment 2. Volumes represent means of lung lesion scores and standard error bars, different lowercase characters show significant differences between groups, a significant difference was observed between IN/(IM), (IM)/IM groups and other three groups, in macro-pathological scores and histopathological scores (HE and IHC). (A) means of macro-pathological lung lesion scores; (B) means of histopathological lesion scores (HE); (c) means of histopathological lesion scores (IHC).



CHAPTER 4. ALPHAVIRUS REPLICON PARTICLES OF DELTA1 SUBTYPE OF INFLUENZA A VIRUS: VACCINE DOSE DETERMINATION, HEMAGGLUTININ INHIBITION AND CHALLENGE RESPONSES

Abstract

Alphavirus recplicon particle (RP) is a replication deficient virus vector expressing genes of interest. RP expressing influenza virus hemagglutinnin (HA) proteins have been developed as an influenza A virus (IAV) vaccine candidate for swine use. HA RP monomers of delta1 H1N2 cluster and H3N2 strains were administered to pigs intramuscularly by a prime/boost vaccination respectively. The immunogenicity of these RPs at varying doses [1×10^7 (infectious unit) IU to 5×10^5 IU], was evaluated in this study. Two H3N2 RP vaccinated groups with 10^6 IU or 5×10^5 IU RP as negative control, and two delta1 RP vaccinated groups with 10^6 IU or 5×10^5 IU RP were all challenged with delta1 IAV virus homologous to delta1 RP. RPs at 5×10^5 IU or higher doses tested in this study were sufficient to induce hemagglutinnin inhibition (HI) detectable antibodies. HI titers were greater at increasing vaccine doses. Delta1 RP 10^6 IU or 5×10^5 IU doses, which induced HI antibodies with mean titers equal or lower than 20, partially prevent homologous infection by eliminating virus replication in lung on day 5 post challenge. However, compared with H3N2 RP vaccinated pigs, decreased lung lesions observed in delta1 RP vaccinated pigs were not statistically significant.

Introduction

Reassortment of H3N2 subtype with hemagglutinin (HA) and neuraminidase (NA) from human influenza A virus (IAV) lineages H1N1 and H1N2 subtypes forming huH1N1 (δ2 or delta2) and huH1N2 (δ1 or delta1) have been reported as spreading in US swine herds (Vincent et al., 2009; Lorusso et al., 2011). According to data from the University of Minnesota Veterinary Diagnostic Laboratory, of all IAV received isolates from swine in 2010, 18% were huH1N2 and 9% were huH1N1 (Pfizer Inc., 2012). Serologically, there is moderate to good HA antibody-antigen cross-reactivity between classical H1 and reassortant H1cluster β and H1cluster γ, but limited cross-reactivity among cluster β, γ and δ, or within δ cluster (Vincent et al., 2008a; Lorusso et al., 2011).

Vaccination is an effective way to control IAV infection to pigs. Traditional inactivated IAV vaccine has been the only commercially available vaccine for swine use for almost 20 years (Platt et al., 2011). Limited cross protection is the major obstacle of controlling IAV by vaccination strategies (Vincent et al., 2010). The most widely used strategy to prevent newly emergent IAV strains is the adding of new strains into vaccines. However, mismatch of inactivated monovalent vaccine strains with challenge virus strains may enhance disease severity including pneumonia and clinical syndromes (Vincent et al., 2008b; Gauger et al., 2011). Autogenous inactivated vaccines have been used in increasing numbers of swine herds (Draayer, 2004; USDA, 2007; Ma and Richt, 2010) . The development of autogenous vaccines normally takes three months to manufacture a vaccine specific to the prevalent strain in a herd, and this method is much more rapid than updating

commercial inactivated vaccine (R. L. Vander Veen, Harrisvaccines Inc., personal communication).

The Alphavirus recplicon particle (RP) system consists of a replication deficient vector expressing a gene of interest with high efficiency and has been employed to develop IAV vaccines (Erdman et al., 2010). An IAV RP vaccine can be produced within six weeks of receiving virus samples or sequences, (R. L. Vander Veen, Harrisvaccines Inc., personal communication). Such rapid speed compared with inactivated autogenous vaccines is a big advantage of RP based vaccines. In addition, because they only express the HA proteins of IAV, RP vaccines only induce antibodies against HA qualifying these vaccines as a Differentiating Infected from Vaccinated Animals (DIVA) vaccine (Vander Veen et al., 2012a). Since RPs are replication deficient, vaccine safety is not a concern. There have been no indications of vaccine spreading to the environment or to un-vaccinated pigs in previously published studies (Vander Veen et al., 2012a). RPs expressing HA of pandemic H1N1 subtype (pH1N1 RP) or cluster IV H3N2 subtype (H3N2 RP) were shown to protect against homologous challenge by significantly reducing virus shedding and lung lesions (Vander Veen, 2011;Vander Veen et al., 2012b). Thus, all above features support RP based vaccines as autogenous vaccine candidates.

An RP expressing HA of delta1 subtype (delta1 RP) was developed recently. In this study, the immunogenicity of delta1 RP and H3N2 RP at lower doses than previously evaluated were determined (Vander Veen, 2011; Vander Veen et al., 2012b) along with the efficacy of delta1 RP protecting pigs against homologous challenge.

Methods and Material

Replicon particle vaccines and influenza virus preparation

RP expressing influenza virus HA proteins were produced as described previously (Vander Veen, 2011). Briefly, HA genes of a cluster IV H3N2 subtype isolate (H3N2) and a cluster delta1 subtype strain of influenza virus were sequenced, amplified with PCR and cloned into a plasmid with using restriction enzyme sites that were incorporated into the PCR oligonucleotide primers. The plasmid also encodes DNA sequences of non-structural proteins of the alphavirus TC-83 strain. HA genes were inserted downstream of an alphavirus 26S promoter (Kamrud et al., 2007). The plasmid was transfected into *Escherichia coli* (*E.coli*) and amplified. Purified plasmid was linearized and transcribed into RNA to create the replicon. Replicon RNA and helper RNAs were electroporated into VERO cells. Eighteen hours later, RPs were harvested from VERO cells and purified with sucrose (Kamrud et al., 2007). RP concentration was titrated on VERO cells as described following. Indirect immunofluorescent assay (IFA) was performed on VERO cells inoculated with RPs to visualize infected cells. Infectious units (IU) were determined by observed numbers of infected cells. Individual RPs were diluted to specific concentration with diluent composed of 60% sucrose, 1% normal swine serum (NSS) and 39% phosphate buffered saline (PBS).

An influenza virus delta1 strain homologous to the strain used to design delta1 RPs, was propagated on MDCK cells and purified, then stored as stock solutions at -80 °C (Szretter et al., 2006). Confluent MDCK cells in T175 flasks were inoculated with 1 ml 10^5 $TCID_{50}$/ml Delta1virus and minimum essential medium (MEM) complemented with 1%

Antibiotic-Antimycotic 100X (GIBCO® #15240, Gand Island, NY) and 2ug/ml TPCK Trypsin (Thermo Fisher Scientific Inc. #20233, Rockford, IL). When all cells detached from the flasks, the flasks were frozen at -80°C and thawed to lyse MDCK cells. Medium with virus and cell lysate was centrifuged at 4000 rotations per minute (rpm) for 45 minutes at 4°C. The supernatant was purified through a 0.45μm filter. The filterate was titrated on MDCK cells and used as the challenge virus.

Animal studies

Thirty-five three week old piglets were purchased from a commercial sow farm. Upon arrival, pigs were weighed and randomly assigned to 7 groups with 5 pigs per group blocked by weight (Table 1). Ceftiofur (Excede® for swine by Pfizer Animal Health, New York, NY) was injected into all pigs per labeled dose of to prevent bacterial infection. Serum samples were collected and evaluated by commercial ELISAs for influenza A NP protein (IDEXX AI multiS-Screen Ab Test by IDEXX Laboratories, Westbrook, ME) and porcine reproductive and respiratory syndrome virus (PRRSV) NP protein (HerdChek X3 by IDEXX Laboratories, Westbrook, ME). After one-week of acclimatization, all pigs began a series of 2 ml intramuscular (IM) prime/boost (day 0/day 21) vaccinations three week apart. Each group was vaccinated with different RPs of varying doses. On day 42, H3-e6, H3-5e5, δ1-e6, and δ1-5e5 groups were challenged intratracheally with 10 ml of 10^4 $TCID_{50}$/ml delta1 strain influenza virus homologous to delta1 RP source virus. H3 RP vaccinated groups served as challenge control in post challenge study and pigs in all the other groups were euthanized on day 42. Serum samples were collected weekly from day of boost vaccination (day 21) to day of challenge (day 42) or/and during necropsy (day 45). Rectal temperatures were measured

and clinical observation scores were assigned to challenged pigs daily from day 42 until necropsy (day 45). One pig in δ1-5e5 group died prior to challenge. Challenged pigs were euthanized at 5 days post challenge (dpc) and necropsied. Macro-pathological lung lesions were observed and scored (0 to 100) blindly by a board-certificated pathologist based on weighted proportions reflecting the estimated total percentage of gross lung lesions to whole lung volume (Halbur et al., 1995). Bronchoalveolar Lavage (BAL) samples and lung tissue samples were collected from each lobe.

Replicon particle titration and indirect immunofluorescence assay

To titrate RPs, VERO cells were seeded on 48 well plates and incubated overnight at 37 °C and 5% CO_2. Tenfold serial dilutions were made to RPs with MDCK grown medium as diluent. Confluent VERO cells were inoculated with the serial dilutions of RP and incubated for 24 hours. VERO cells were fixed by ice-cold acetone/methanol (1:1) for 5-8 minutes followed by three washes with Imidazole Buffered Saline with Tween 20 (KPL # 50-63-00, Gaithersburg, MD). Goat antibodies specific to the NSP protein of Venezuela equine encephalitis virus, was diluted by 1:200 with COSMIC Calf Serum (CCS)/PBS (1:1) and were coated on MDCK cells for 1 hour at 37 °C. Alexa Flour® 488 donkey anti-goat IgG (H+L) (Invitrogen™ #A11055, Grand Island, NY) antibodies were then incubated on cells after three washes. After one hour of incubation at 37 °C followed by three more washes, infected cells were counted using an inverted fluorescent microscope equipped with a 488nm wave length filter to determine infectious unit (IU) of RP.

Clinical evaluation

Clinical signs, correlated to influenza infection and rectal temperatures, were evaluated daily starting on the day of challenge (day 42) until necropsy (day 45). Coughing, respiratory rate, and behavior were assigned the following scores. Coughing scoring was: 1=none, 2=soft or mild intermittent cough, 3=harsh or severe, repetitive cough, 4=dead. Respiratory scoring was: 1=normal, 2=panting/rapid, 3=dyspnea, 4=dead. Behavior scoring was: 1=normal, 2=mild lethargy, 3=severe lethargy or recumbancy, 4=dead. Behavior score, coughing score, and respiratory score were summed up as a clinical score of each pig on each day.

Live virus isolation

BAL samples in MEM with 1% Antibiotic-Antimycotic, were stored at -80 °C until use. Medium was centrifuged at 3500 rpm for 25 minutes and the supernatant was collected for virus isolation. Virus isolation protocol was similar to RP titration. MDCK cells seeded on 96 well plates were inoculated by influenza virus along with 2μg/ml TPCK trypsin (Thermo Fisher Scientific Inc. #20233) and incubated for 5 days until no more cytopathic effect (CPE) emerged. IFA was performed with 1:1000 dilution of mouse anti-NP protein of influenza A (EMD Millipore Corporation #MAB8257, Billerica, MA) followed by 1:1000 dilution of Alex Fluor® 488-conjugated donkey anti-mouse IgG (H&L) antibodies (Invitrogen™ #A21202, Grand Island, NY). The Reed-Muench equation was used to determine virus titers (Reed and Muench, 1938).

Hemagglutin inhibition assay

Serum samples were sent to University of Minnesota veterinary diagnostic laboratory (UM VDL) to perform hemagglutinin inhibition (HI) assay. The HI assay protocol was similar to one previously described (Vander Veen et al., 2009). Homologous HA antigens matched to different RPs, were used in the HI assay. The titers of HI antibodies induced by delta1 RP against a heterologous strain in the same cluster and a heterologous cluster delta2-Pfizer XP 31 strain, were also evaluated. Titers of HI antibodies in serum against specific HA proteins were determined based on two fold dilutions with a minimum detectable titer of 10.

Pathological evaluation

Lung tissue samples were placed into 10% formalin. Twenty-four hours later, processed samples were transferred to 70% ethanol and stored at room temperature until sent to Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). Hematoxylin and eosin (HE) staining and influenza virus A specific immunohistochemistry (IHC) staining were performed on each sample (Vincent et al., 1996). HE stained samples were scored 0 to 3 reflecting the severity of bronchial epithelial injury according to the method by Vincent et al. (Vincent et al., 2007). IHC stained samples were scored 0 to 3 depending on amount of influenza virus antibody stained cells using following criteria: 0= no staining, 1= mild staining, 2= moderate staining, 3= marked staining. A board-certified veterinary pathologist did all scoring work blind to pig treatment.

Statistics

Single factor analysis of variance (ANOVA) was used to evaluate the significant difference of data. P-value of statistic significant difference was <0.05 two sided. Linear regression was used to evaluate the relationship of HI titers and different doses. Linear regression between delta1 RP doses (x) and transferred HI titers against homologous strain (y) was tested. Original HI titers (z) were transferred as following: $y=\log_2(2z/10)$.

Results

Clinical evaluation

The behavior of all challenged pigs was normal; pigs were aware, active and not lethargic. Mild respiratory syndromes, correlated to influenza, were observed, including mild coughing, panting, and rapid breathing. Means of clinical scores for each group on each observed day were not significantly different among groups (range from 3±0 to 3.4±0.2). Most pigs were not observed with pyrexia with the highest temperature (105 °F) only observed at 1 time-point in one pig. At all time-points, the means of body temperatures were not significantly different among all groups.

Live virus isolation

No virus was isolated from BAL samples of δ1-e6 and δ1-5e5 groups (Figure 1). All pigs that received H3 RP had detectable levels of live virus in BAL samples. Mean virus titers in H3 RP vaccinated groups with 1×10^6 IU RP (4±0.2) and 5×10^5 IU RP (4.5±0.4) were not significantly different.

Hemagglutinin inhibition assay

In H3N2 RP vaccinated groups upon boost vaccination (day 21), no pig had developed homologous HI antibodies with a titer higher than 20. On day 27, all doses (5×10^6 IU, 10^6 IU and 5×10^5 IU) induced HI antibodies against homologous virus; geometrical titer means of each group were at least 160 (Figure 2A). On day 40, HI titers of all H3N2 RP vaccinated groups decreased, but fourteen of fifteen pigs had HI titers at least 40. The H3-5e6 group HI titer (mean titer: 121) was significantly greater than the other 2 groups (Figure 2A). HI titers against delta1 virus were tested on serum samples from H3-e6 and H3-5e5 vaccinated groups on day 40. No pigs in H3-5e6 or H3-5e5 vaccinated group developed detectable HI antibodies against delta1 strain (data not shown).

In delta1 RP vaccinated groups against a homologous or heterologous δ1 strain in the same cluster, δ1-e7 and δ1-5e6 groups developed HI antibodies with means titer ≥46, while δ1-e6 (mean titer: 20) and δ1-5e5 (mean titer: 11) groups developed lower HI antibodies titers against homologous strain or heterologous δ1 strain (Figure 2B). HI antibodies, induced by all doses of delta1 RP, had poor cross-reactivity with delta2 sub-cluster with mean titers all below 10 (Figure 2B). A linear regression exists between delta1 RP doses (x) and transferred HI titers against homologous strain (y): $y = 0.1274x + 1.6099$. $R^2 = 0.5994$ (Appendix 1).

Pathological evaluation

Upon macro-pathological examination, all pigs (n=10) vaccinated with H3N2 RP developed lung lesions. Mean of H3-e6 group was 9.2±1.2 and mean of H3-5e5 group was

12±3.9. Three of five pigs in δ1-e6 group (5.2±2.6), and only one of four pigs in δ1-5e5 group (4.8±4.8) developed lung lesions (figure 3A; Appendix 2). In HE staining, all pigs, except one in δ1-e6 group, were observed to possess histopathological lesions with different severity (Appendix 2). Mean of HE scores of H3-5e6 group was 1.8±0.2, H3-5e5 group was 2±0.2, δ1-e6 group was 1.3±0.4 and δ1-5e5 group was 1.5±0.5 (figure 3B). However, in IHC staining, two of five samples in H3-5e5 group (0.4±0.2), only one of five samples in each of H3-e6 group (0.2±0.2) and δ1-e6 group (0.2±0.2) were observed with strained virus in lung tissues, while virus was not observed in any of the four samples in δ1-5e5 group (figure 3C; Appendix 2).

In macro-pathological scores and HE scores, delta1 RP vaccinated groups had lower means than groups vaccinated with H3N2 RP. In IHC scores, groups vaccinated with delta1 RP had mean scores at the same or lower levels than H3N2 group. Higher doses of H3N2 RP vaccination resulted in lower lung lesion scores with all staining methods after delta1 challenge. However, none of these differences were statistically significant (Figure 3A,B&C).

Discussion

The RP system can be employed to express variant HA proteins of influenza strains from field isolates (Vander Veen et al., 2012b). This study provides an efficacy parameter indicating the lowest efficient dose of RP-expressing HA genes from different influenza strains. These finding will aid in determination of the dose of RP needed in future studies. With 5×10^5 IU of RPs expressing different HAs (H3N2 or delta1), nine of ten pigs tested had

homologous HI titer of as least 40 on day 27, and eight of nine pigs had detectable homologous HI antibodies (≥20) on day 40. Virus replication in lungs was completely inhibited in pigs vaccinated by 5×10^5 IU delta1 RP at 5 dpc and lung lesions were reduced compared with control pigs. Thus 5×10^5 IU was a sufficient dose to eliminate virus replication in pigs. In addition, this is the first reported study to evaluate efficacy of delta1 RP in protecting pigs against homologous challenge. In contrast to a high dose (10^8 IU) used in previous studies to evaluate HA RP protection efficacy (Vander Veen, 2011;Vander Veen et al., 2012b), this study demonstrates a much lower dose of RP that still provides protection to pigs, which shows the strong immunogenicity of RP based IAV vaccines. In addition, the serological results show antibodies induced by delta1 RP have robust cross-reactivity with heterologous strain within the same sub-cluster.

Clinical syndromes and rectal temperatures, of pigs experimentally challenged with IAV were not significantly different from non-challenged pigs in this study and in other experimental challenge studies (Ma et al., 2011;Vincent et al. 2008b). Therefore, whether homologous RP vaccine significantly prevented influenza clinical syndromes in pigs was not demonstrated in this study. However, in a previous study, H3 RP was shown to reduce fever significantly compared with non-vaccinated pigs following homologous challenge (Vander Veen et al., 2012b).

With the presence of HI antibodies with mean titer equal or lower than 20, virus replication in lungs was completely inhibited at 5 dpc, however, pneumonia was not. In chapter 3, following one dose IM vaccination with RP, virus replication was inhibited in the presence of low HI titer prior to challenge, agreeing with this study. It was previously

reported that high titers of homologous HI antibodies (titer> 100) completely inhibit virus replication (Heinen et al., 2001; Wesley et al., 2004; Vander Veen et al., 2009). Here, we reported that HI titer of 20 or lower inhibits virus replication as well. The capacity of the RP vaccine to stimulate memory T cells has been shown in Vander Veen et al. (2011). Memory cells circulating in serum may be stimulated by antigens rapidly. Thus, a high HI titer may not be necessary to prevent homologous influenza infection in pigs.

Following two IM vaccinations, all doses of H3N2 RP evaluated in this study were sufficient to induce high titers (≥160) of homologous HI antibodies on day 27 and the antibodies lasted at least until day 40. For delta1 RP, by rising the vaccine dose, HI antibody titer could be increased and there was a linear regression relationship observed between the dose and the titer. In contrast, circulating HI antibodies against H3 antigen upon challenge, did not have measurable cross-reactivity with challenge virus in HI assay. However, all pathological scores in H3-e6 group were lower than H3-5e5 group. Gauger et al. has shown that inactivated delta1 vaccine enhanced disease including pneumonia and clinical syndromes following H1 homosubtypic, heterologous pandemic H1N1 challenge (Gauger et al., 2011). Vincent et al. showed inactivated classical H1N1 vaccine enhanced pneumonia following delta1 challenge (Vincent et al., 2008b). There was no cross-reaction between the vaccine-induced HI antibodies to challenge antigen, when pneumonia was enhanced in the study by Gauger et al. (2011). In contrast, commercial multivalent vaccine-induced HI antibodies which had low or unmeasurable reactivity to challenge antigen, but provided partial heterologous protection to pandemic H1N1 challenge (Vincent et al., 2010). Therefore, cross-reactivity of HI antibodies may not indicate the cross protection efficacy of vaccines. In

addition, modified live virus vaccine did not enhance disease, but provided partial protection to heterologous challenge (Vincent et al., 2007). Because of the lack of a non-vaccinated challenge group as a control, whether H3N2 RP reduced or enhanced disease severity could not be demonstrated. Evaluating whether RP based vaccine protects heterologous strain challenge *in vivo* in future studies will help to estimate the risk of RP vaccine when the RP vaccine is a mismatch with the challenge virus. It will also help to determine whether the disease enhancement by mismatch vaccine is related to virus strains, certain virus proteins or vaccine types.

To conclude, this study shows that prime/boost RP at 5×10^5 IU or higher doses tested were sufficient to induce detectable HI antibody. HI titers could be increased by increasing vaccine doses. Delta1 RP at 10^6 IU and 5×10^5 IU, which induced HI antibodies with mean titers equal to or lower than 20, partially protected homologous infection by eliminating virus replication in lungs at 5dpc. However, compared with H3N2 RP vaccinated pigs, decreased lung lesions observed in delta1 RP vaccinated pigs were not statistically significant.

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Table 1. Animal study protocol

Group name	Pig numbers	HA subtype expressed by RP[1]	RP Dose[2]	Challenge[3]	Euthanasia day
H3-5e6	5	Cluster IV H3N2	5×10^6 IU		42
H3-e6	5		1×10^6 IU	×	47
H3-5e5	5		5×10^5 IU	×	47
δ1-e7	5	Delta 1 huH1N2	1×10^7 IU		42
δ1 -5e6	5		5×10^6 IU		42
δ1-e6	5		1×10^6 IU	×	47
δ1–5e5	5		5×10^5 IU	×	47

[1] Each pig received the same vaccine intramuscularly in both prime and boost vaccination. [2] Each dose was in 2ml volume administered intramuscularly. [3] All challenged pigs were challenged on day 42 with the same virus homologous to δ1 RP.

Figure 1. Virus isolation titers from BAL samples at 5 dpc, different characters representing significant differences.

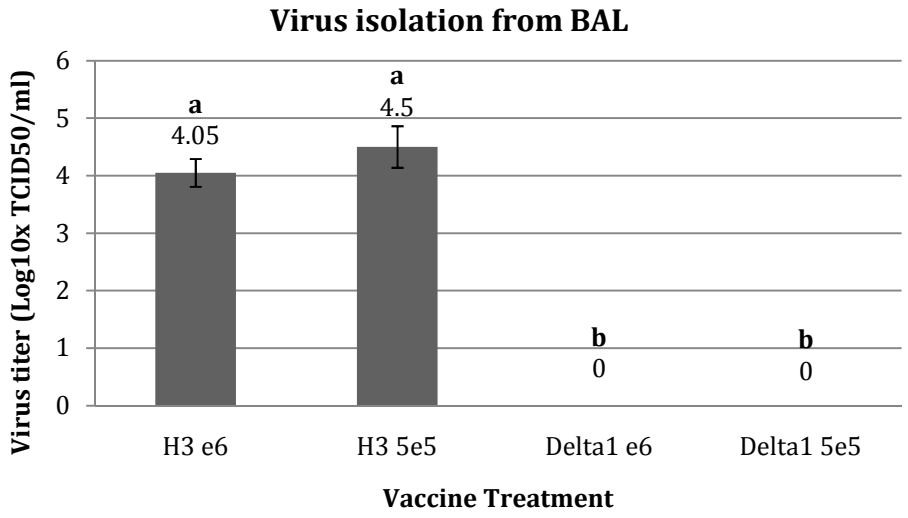
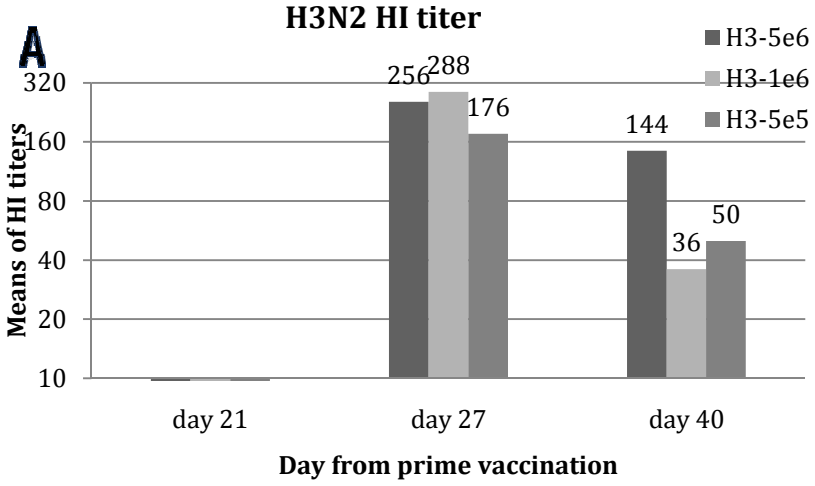


Figure 2. (A) HI titers of H3N2 RP vaccinated groups against homologous H3N2 antigen, different characters representing significant differences, uppercase characters representing day 27 and lowercase characters representing day 40; (B) HI titers of serums samples from delta1 RP vaccinated groups on day 40 against different antigens. Homo: homologous delta1 antigen; hetero d1: heterologous delta1 antigen; d2: heterologous delta2 antigen.



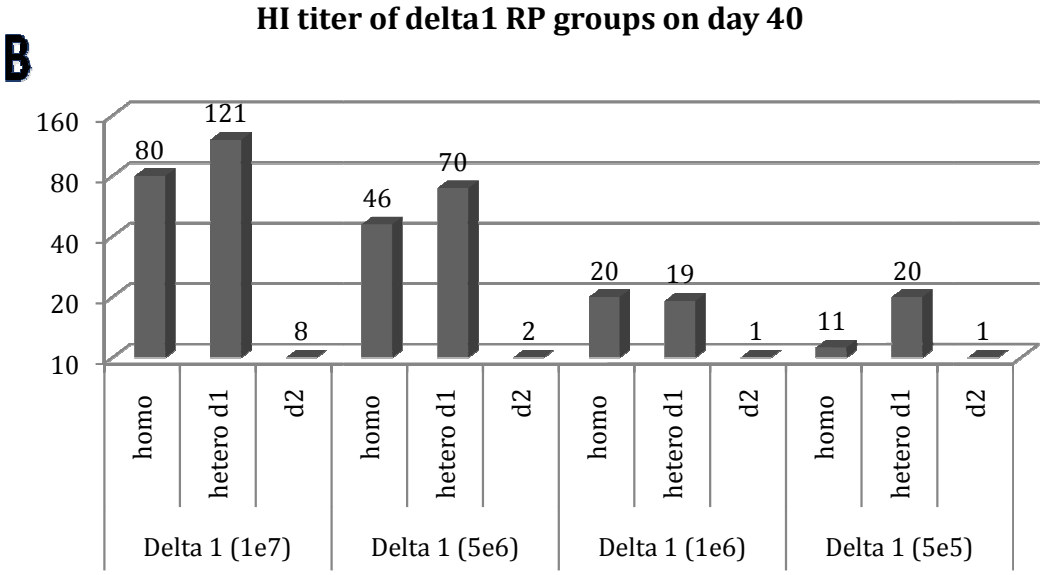
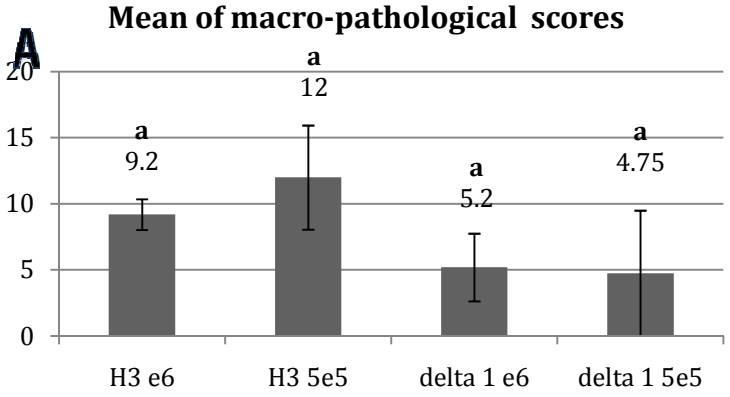
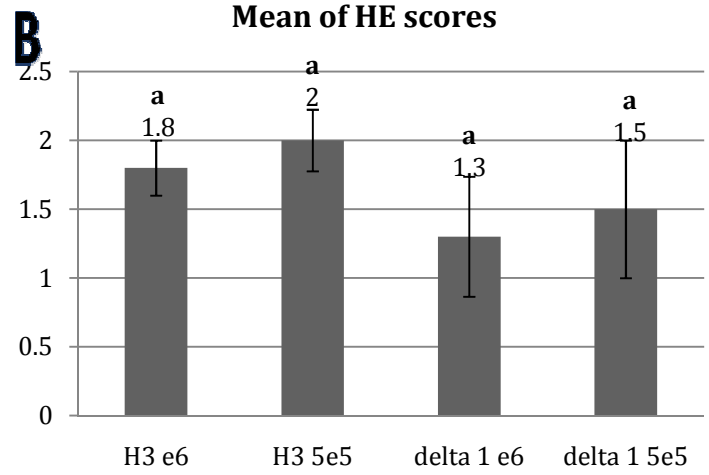
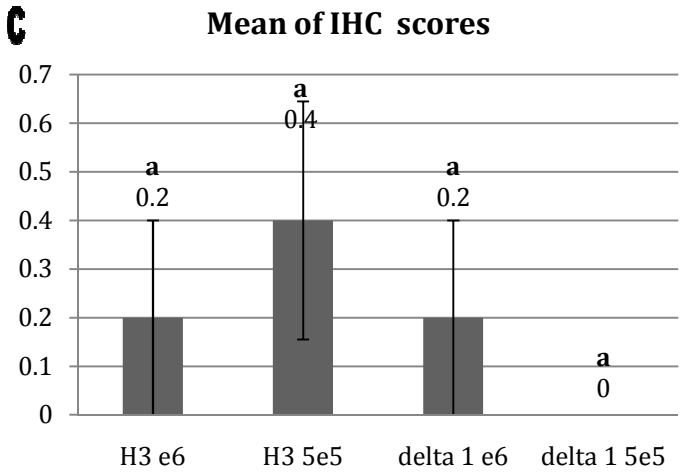


Figure 3. Different characters representing significant differences. (A) Means of macro-pathological scores of each group; (B) means of HE lesion scores of each group; (C) means of IHC scores of each group.







CHAPTER 5. GENERAL CONCLUSIONS

Various methods of vaccination of pigs for influenza A virus (IAV) were reviewed. Currently, there are two vaccination schemes can be used for protection against IAV. These are USDA licensed commercial vaccine and USDA licensed autogenous vaccines. Since it takes over one year to change the subtypes present in a commercial vaccine, the autogenous vaccine which can be produced within 3 to 4 months currently offers an alternative vaccination method. However, the USDA does not require efficacy or potency to be conducted with autogenous vaccines.

Different administering routes with IAV replicon particle (RP) have been tested. Significant protection was provided by one dose intramuscular (IM) RP vaccination. The result also demonstrated that the protection provided by RP was induced as early as three weeks post prime vaccination. Intranasal/IN administering RP did not protect pigs against homologous IAV challenge. A different method of aerosolization or nebulization RP droplet may results in better protection.

Herein, studies were conducted for the first time with a RP prepared against IAV delta1 sub-cluster. Three doses were compared, and the lower dose of 5×10^5 IU RP partially protected pigs. In addition, it was found that 5×10^5 IU RP induced hemagglutinin (HI) antibodies with a mean titer lower than 20, and with the presence of such level of HI antibodies, virus replication was inhibited in pigs.

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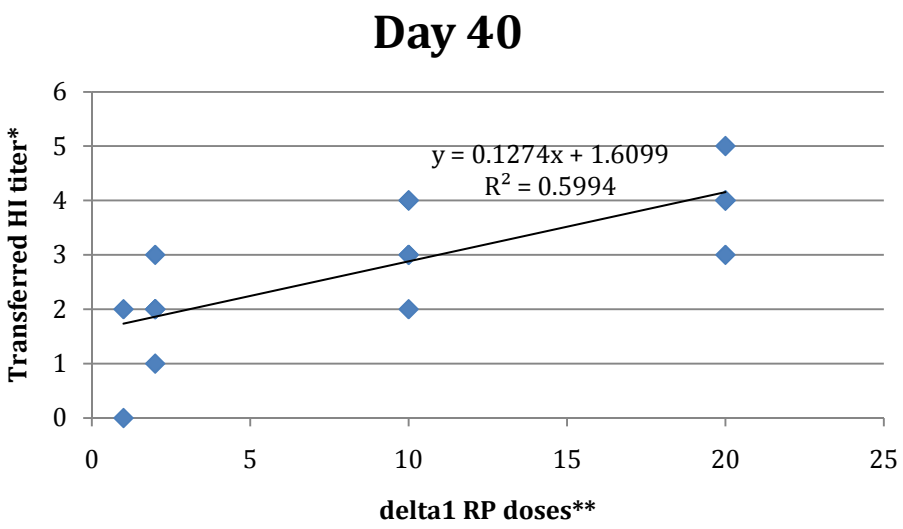
APPENDIX 1

Linear regression of delta1 RP doses (x-axis) and modified HI titers* (y-axis) against homologous δ1 antigen.



* Transferred HI titer= log2 (2z/10); z=original HI titer. ** 5×10^5 IU was defined as one dose.

APPENDIX 2

A summary of pathological scores and virus isolation titers of each pig of studies of Chapter 4

Vaccine	Heterologous delta 1 HI titer (day of challenge)	Macro-pathological Score	HE score	IHC Score	BAL virus titer
H3 (1e6)	Neg	9	1	0	3.75
H3 (1e6)	Neg	10	2	1	4.75
H3 (1e6)	Neg	8	2	0	4.5
H3 (1e6)	Neg	13	2	0	3.75
H3 (1e6)	Neg	6	2	0	3.5
H3 (5e5)	Neg	3	2	1	4.5
H3 (5e5)	Neg	26	2.5	1	3.25
H3 (5e5)	Neg	10	1.5	0	4.75
H3 (5e5)	Neg	7	1.5	0	5.5
H3 (5e5)	Neg	14	2.5	0	4.5
Delta (1e6)	40	4	1	0	0
Delta (1e6)	Neg	9	2.5	0	0
Delta (1e6)	40	13	2	1	0
Delta (1e6)	20	0	1	0	0
Delta (1e6)	80	0	0	0	0
Delta (5e5)	20	19	3	0	0
Delta (5e5)	20	0	1	0	0
Delta (5e5)	20	0	1	0	0
Delta (5e5)	20	0	1	0	0